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(54) **METHODS FOR DETERMINING THE PROGNOSIS FOR CANCER PATIENTS USING TUCAN**  
 VERFAHREN ZUR BESTIMMUNG DER PROGNOSE VON KREBSPATIENTEN MITTELS TUCAN  
 METHODES PERMETTANT D'ETABLIR UN PRONOSTIC POUR DES PATIENTS ATTEINTS D'UN  
 CANCER SUR LA BASE DU NIVEAU DE TUCAN

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- **PATHAN N. ET AL.: 'TUCAN, an antiapoptotic caspase-associated recruitment domain family protein overexpressed in cancer' JOURNAL OF BIOLOGICAL CHEMISTRY vol. 276, no. 34, 24 August 2001, pages 32220 - 32229, XP002977129**

**Description****BACKGROUND OF THE INVENTION**

5 **[0001]** This invention relates generally to colon cancer and, more specifically, to biomarkers that can be used to diagnose or prognose cancer.

**[0002]** 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 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  
10 whose cancers are found early also are less likely to die from these cancers than are those whose cancers are not found until symptoms appear.

**[0003]** 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 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.  
15 Unfortunately, some tumor markers also can be detected in significant amounts in 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.

**[0004]** Another important use for tumor markers is for monitoring patients being treated for advanced cancer. 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.  
20

**[0005]** 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 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  
25 examination or surgery. Knowledge of the likelihood of metastasis in a 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.

**[0006]** WO 01/30813 discloses CARD-8, which is a synonym for TUCAN; it also discloses, that methods of detecting CARD-8 can be used as prognostic assays. WO 01/22920 discloses that a variant of TUCAN (SEQ ID NOs:2105 and 6382) is enhanced in human colon and colon cancer tissue.  
30

**[0007]** Thus, there exists a need for identification of 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**

**[0008]** The invention provides methods for determining a prognosis for survival for a colon cancer patient. A first method involves (a) measuring a level of 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 level of TUCAN lower than the  
40 reference level in the sample correlates with increased survival of the patient.

**[0009]** Another method for determining a prognosis for survival for a cancer patient involves in addition to the steps of the first method (a) measuring levels of one or more biomarkers selected from the group consisting of cIAP2, Apaf1, Bcl-2 and Smac in the neoplastic cell-containing sample from the cancer 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  
45 biomarkers, wherein a level of TUCAN lower than the reference level and a level of any of Apaf1, Bcl-2 or Smac higher than the reference level, or a level of TUCAN and a level of cIAP2 lower than the reference level, in said sample correlate with increased survival of said patient.

**[0010]** A further method of determining a prognosis for survival for a cancer patient involves in addition to the steps of the first method classifying the patient as belonging to either a first or second group of patients, wherein the first group  
50 of patients having levels of TUCAN lower than the reference level is classified as having an increased likelihood of survival than the second group of patients having levels of TUCAN higher than the reference level.

**[0011]** The invention also provides a method for monitoring the effectiveness of a course of treatment for a patient with colon cancer. The method involves (a) determining a level of TUCAN in a neoplastic cell-containing sample from the cancer patient prior to treatment, and (b) determining the level of TUCAN in a neoplastic cell-containing sample from  
55 the patient after treatment, whereby comparison of the TUCAN level prior to treatment with the TUCAN level after treatment indicates the effectiveness of the treatment wherein a decrease in the level of TUCAN after treatment indicates that treatment is effective.

**BRIEF DESCRIPTION OF THE DRAWINGS****[0012]**

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

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

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

Figure 4 shows correlations of biomarker immunostaining data with disease-free (Figure 4A-4P) and overall survival (Figure 4Q-4F-1) for colon carcinoma patients.

15 Figure 5 shows correlations of biomarkers and 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.

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

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

**DETAILED DESCRIPTION OF THE INVENTION**

25 **[0013]** This invention relates to the finding that expression of the CARD domain containing protein, TUCAN (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 colon cancer, either independently, or in combination with other biomarkers.

30 **[0014]** 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, 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 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 surgically treated patients likely to experience colon 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.

35 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.

**[0015]** The prognostic methods of the invention also are useful for determining a proper course of treatment for a patient having colon 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.

40 **[0016]** 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.

55 **[0017]** As shown in Example IV, at a median follow-up of 5 years, 49% of patients with high expression of TUCAN 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 ( $p = 0.00004$ ). 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 likelihood of tumor metastasis and increased likelihood of survival.

5 **[0018]** 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 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, 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, 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.

15 **[0019]** Based on these results, methods are provided for diagnosing neoplastic conditions, prognosing survival of patients suffering from colon 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 colon cancer.

20 **[0020]** 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 ( $p = 0.0002$ ),  $\beta$ -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-4P). Thus, 78% (39/50) of patients H 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.

25 **[0021]** 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.

30 **[0022]** 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.

35 **[0023]** 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.

40 **[0024]** 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. 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  $\beta$ -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  $\beta$ -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.

**[0025]** 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 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 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)).

**[0026]** 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.

**[0027]** 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.

**[0028]** 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.

**[0029]** 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 be manipulated to simulate tumor cells, or can be manipulated in any other manner which yields expression levels which accurately determine the reference level.

**[0030]** The reference level can also be determined by comparison of the level of a biomarker, such as TUCAN, in 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 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 reference level can then be made based

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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 biomarker.

**[0031]** 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 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 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.

**[0032]** 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 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.

**[0033]** As used herein, the term "cancer" is intended to mean a class of diseases characterized by the 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 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 cancer; leukemia and melanoma. By exemplification, a list of known cancers is provided below in Table 1.

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

5 Lymphoid Neoplasms  
 Myeloid Neoplasms  
 Histiocytoses  
 10 Precursor B lymphoblastic  
 leukemia/lymphoma (ALL)  
 Precursor T lymphoblastic  
 15 leukemia/lymphoma (ALL)  
 Chronic lymphocytic  
 leukemia/small lymphocytic  
 20 lymphoma (SLL)  
 Lymphoplasmacytic lymphoma  
 Mantle cell lymphoma  
 Follicular lymphoma  
 25 Marginal zone lymphoma  
 Hairy cell leukemia  
 Plasmacytoma/plasma cell  
 30 myeloma  
 Diffuse large B-cell  
 lymphoma  
 35 Burkitt lymphoma  
 T-cell chronic lymphocytic  
 leukemia  
 40 Large granular lymphocytic  
 leukemia  
 Mycosis fungoids and sezary  
 syndrome  
 45 Peripheral T-cell lymphoma,  
 unspecified  
 Angioimmunoblastic T-cell  
 50 lymphoma  
 Angiocentric lymphoma  
 (NK/T-cell lymphoma)  
 55 Intestinal T-cell lymphoma

Adult T-cell  
 leukemia/lymphoma  
 Anaplastic large cell  
 lymphoma  
 Hodgkin Diseases (HD)  
 Acute myelogenous leukemia  
 (AML)  
 Myelodysplastic syndromes  
 Chronic Myelofroliferative  
 Disorders  
 Chronic Myelogenous  
 Leukemia (CML)  
 Polycythemia Vera  
 Essential Thrombocytosis  
 Myelofibrosis with Myeloid  
 Metaplasia  
 Hemangioma  
 Lymphangioma  
 Glomangioma  
 Kaposi Sarcoma  
 Hemanioendothelioma  
 Angiosarcoma  
 Hemangiopericytoma  
  
**HEAD & NECK**  
 Basal Cell Carcinoma  
 Squamous Cell Carcinoma  
 Ceruminoma  
 Osteoma  
 Nonchromaffin Paraganglioma  
 Acoustic Neurinoma  
 Adenoid Cystic Carcinoma  
 Mucoepidermoid Carcinoma  
 Malignant Mixed Tumors  
 Adenocarcinoma

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

**THE PANCREAS**

Adenocarcinoma  
 5 Cystadenocarcinoma  
 Insulinoma  
 Gastrinoma  
 10 Glucagonamoa

**THE KIDNEY**

15 Renal Cell Carcinoma  
 Nephroblastoma (Wilm's  
 Tumor)

**THE LOWER URINARY TRACT**

20 Urothelial Tumors  
 Squamous Cell Carcinoma  
 25 Mixed Carcinoma  
 Adenocarcinoma  
 Small Cell Carcinoma  
 30 Sarcoma

**THE MALE GENITAL TRACT**

35 Squamous Cell  
 CarcinomaSarcinoma  
 Speretocytic Sarcinoma  
 40 Embyonal Carcinoma  
 Choriocarcinoma  
 Teratoma  
 45 Leydig Cell Tumor  
 Sertoli Cell Tumor  
 Lymphoma  
 50 Adenocarcinoma  
 Undifferentiated Prostatic  
 Carcinoma  
 55 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	BONES, JOINTS, AND SOFT
	Paget's Disease	TISSUE TUMORS
5	Carcinoma	Multiple Myeloma
	Insitu Carcinoma	Malignant Lymphoma
	Invasive Carcinoma	Chondrosarcoma
10		Mesenchymal Chondrosarcoma
	<b>THE ENDOCRINE SYSTEM</b>	Osteosarcoma
	Adenoma	Ewing Tumor (Ewing Sarcoma)
15	Carcinoma	Malignant Giant Cell Tumor
	Meningioma	Adamantinoma
	Craniopharyngioma	Malignant Fibrous
20	Papillary Carcinoma	Histiocytoma
	Follicular Carcinoma	Desmoplastic Fibroma
	Medullary Carcinoma	Fibrosarcoma
25	Anaplastic Carcinoma	Chordoma
	Adenoma	Hemangioendothelioma
	Carcinoma	Emangispericytoma
30	Pheochromocytoma	Liposarcoma
	Neuroblastoma	Malignant Fibrous
	Paraganglioma	Histiocytoma
	Pineal	Rhabdomyosarcoma
35	Pineoblastoma	Leiomyosarcoma
	Pineocytoma	Angiosarcoma
40	<b>THE SKIN</b>	<b>NERVOUS SYSTEM</b>
	Melanoma	Schwannoma
	Squamous cell carcinoma	Neurofibroma
45	Basal cell carcinoma	Malignant Peripheral Nerve
	Merkel cell carcinoma	Sheath Tumor
	Extramammary Paget's Disease	Astrocytoma
50	Paget's Disease of the	Fibrillary Astrocytoma
	nipple	Glioblastoma Multiforme
	Kaposi's Sarcoma	Brain Stem Glioma
55	Cutaneous T-cell lymphoma	Pilocytic Astrocytoma

Pleomorphic  
Xanthorastrocytoma  
5 Oligodendroglioma  
Ependymoma  
Gangliocytoma  
10 Cerebral Neuroblastoma  
Central Neurocytoma  
Dysembryoplastic  
15 Neuroepithelial Tumor  
Medulloblastoma  
Malignant Meningioma  
20 Primary Brain Lymphoma  
Primary Brain Germ Cell  
Tumor

Langerhans Cell

Histiocytosis

25 **THE EYE**  
Carcinoma  
30 Squamous Cell Carcinoma  
Mucoepidermoid Carcinoma  
Melanoma  
Retinoblastoma  
35 Glioma  
Meningioma

40 **THE HEART**  
Myxoma  
Fibroma  
45 Lipoma  
Papillary Fibroelastoma  
Rhasdoyoma  
50 Angiosarcoma  
Other Sarcoma

55 **HISTIOCYTOSSES**

**[0034]** 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.

**[0035]** 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.

**[0036]** 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.

**[0037]** 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.

**[0038]** 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.

**[0039]** The invention relates to the use of TUCAN as a biomarker for prognosing survival and monitoring the effectiveness of a treatment for a colon 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.

**[0040]** 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.

**[0041]** 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 characterized, including Apaf1, Nod1 (CARD4), NAC (DEPCAP), Raidd (CRADD), Cardiak (Rip2, RICK), Bc110 (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 prodomains of specific CARD-carrying caspases, either facilitating or inhibiting protease activation.

**[0042]** 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 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 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 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, 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.

5 **[0043]** 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.

10 **[0044]** 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).

15 **[0045]** 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 whose tumors contained higher levels of TUCAN indeed were more likely to die from their disease, based on retrospective analysis using archival specimens.

20 **[0046]** Therefore, the invention provides a method for determining a prognosis for survival for a colon cancer patient using TUCAN. The method involves (a) measuring a level of 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 levels of TUCAN lower than the reference level in the sample correlate with increased survival of the patient.

25 **[0047]** 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 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 or spread.

30 **[0048]** The methods of the invention for prognosing survival for a colon cancer patient involve measuring the level of one or more biomarkers, at least TUCAN. The level of the biomarker 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.

35 **[0049]** 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 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 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 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 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.

40 **[0050]** In the methods of the invention, a sample can 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 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, urine or stool, is less invasive than collecting a tissue sample.

45 **[0051]** 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 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 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.

**[0052]** Essentially all modes of affinity binding assays are applicable for use in determining a level of TUCAN, or another biomarker polypeptide, such as cIAP2, Apaf1, Smac,  $\beta$ -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.

**[0053]** 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.

**[0054]** 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).

**[0055]** 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).

**[0056]** 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, 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 1992); Borrabeck, *Antibody Engineering*, 2d ed. (Oxford University Press 1995)).

**[0057]** 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 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, for example, the use of labeled secondary antibodies and binding agents.

**[0058]** 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 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 antibody to enhance the sensitivity of the binding assay if desired.

**[0059]** 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 bioluminescent compounds. Specific examples of enzyme labels include horseradish peroxidase (HRP), alkaline phosphatase (AP),  $\beta$ -galactosidase, urease and luciferase.

**[0060]** A horseradish-peroxidase detection system can be used, for example, with the chromogenic substrate tetram-

ethylbenzidine (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 example, which yields a soluble product readily detectable by measuring absorbance at 405 nm. Similarly, a  $\beta$ -galactosidase detection system can be used with the chromogenic substrate o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG), which yields a soluble product 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 oxidation.

**[0061]** 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, 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.

**[0062]** Chemiluminescent as well as bioluminescent detection labels are convenient for sensitive, non-radioactive detection of a biomarker and can be obtained commercially from various sources such as Amersham Lifesciences, Inc. (Arlington Heights, IL).

**[0063]** Alternatively, radioisotopes can be used as detectable labels in the methods of the invention. Iodine-125 is a specific example of a radioisotope useful as a detectable label.

**[0064]** Signals from detectable labels can be analyzed, for example, using a spectrophotometer to detect color from a chromogenic substrate; a fluorometer to detect 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 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 samples can be detected simultaneously.

**[0065]** 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, 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.

**[0066]** A binding agent selective for a biomarker also 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 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 with, for example,  $^{111}$ indium 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, Apaf1, Smac,  $\beta$ -catenin, Bcl-2 or p53. Imaging can be accomplished by, for example, radioimmunoscinigraphy as described by Kahn et al., *supra*.

**[0067]** The level of TUCAN, or another biomarker, such as cIAP2, Apaf1, Smac,  $\beta$ -catenin, Bcl-2 or p53, also can 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 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,  $\beta$ -catenin, survivin or Apaf1 gene, or fragment thereof.

**[0068]** 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 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 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 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 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, Smac,  $\beta$ -catenin, Bcl-2 or p53, can be similarly determined and compared to a suitable reference

level for the particular biomarker.

**[0069]** 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.

**[0070]** 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.

**[0071]** Nucleic acid probes useful for measuring the expression level of a biomarker, such as cIAP2, TUCAN, Apaf1,  $\beta$ -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,  $\beta$ -catenin, Bcl-2, or Smac nucleotide sequences and which are capable of specifically or selectively hybridizing to cIAP2, TUCAN, Apaf1,  $\beta$ -catenin, Bcl-2, or Smac RNA, are useful for hybridization methods.

**[0072]** A reference level is a level a biomarker, such as cIAP2, TUCAN, Apaf1, Smac,  $\beta$ -catenin, of Bcl-2, used to evaluate the level of the biomarker in cancerous cells of a patient. Specifically, when the level of a 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 reference level, the cells will be considered to have a low level of, or underproduction, of the biomarker.

**[0073]** A high level of a biomarker, such as cIAP2, TUCAN, Apaf1, Smac,  $\beta$ -catenin, Bcl-2 or p53, or overproduction of a biomarker gene is related to a level of the biomarker above a determined basal level. Thus, a reference or basal level of a biomarker, such as cIAP2, TUCAN, Apaf1, Smac,  $\beta$ -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 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 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 number and source of samples used to determine reference or basal values.

**[0074]** 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.

**[0075]** 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.

**[0076]** 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 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).

**[0077]** Population-based determination of reference 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 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.

**[0078]** 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. 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 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.

**[0079]** 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.

**[0080]** The reference level can also represent the level of a biomarker protein, such as cIAP2, TUCAN, Apaf1, Smac,  $\beta$ -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).

**[0081]** 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.

**[0082]** 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 colon 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,  $\beta$ -catenin, MIB-1 or another well-known tumor marker, such as the exemplary commercially available tumor markers described below. Furthermore, the use of a combination of TUCAN with one or more biomarkers can provide increased prognostic significance or confidence in a prognostic determination.

**[0083]** Therefore, the invention provides a method for determining a prognosis for survival for a colon cancer patient 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 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 level of TUCAN lower than the reference level a level of any of Apaf1, Bcl-2 or Smac higher than the reference level or a level of TUCAN and a level of cIAP2 lower than the reference level, in said sample correlate with increased survival of said patient.

**[0084]** 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 expression correlates with improved survival, such as any of cIAP2, Apaf1, Bcl-2, Smac, or another known or standard biomarker for colon cancer. There are general diagnostic or prognostic markers useful for multiple types of cancer, such as CA 125, CEA or LDH, diagnostic or prognostic colon cancer markers (for example, sialosyl-TnCEA, CA19-9, or LASA), breast cancer markers (for example, CA 15-2, Her-2/neu and CA 27.29), ovarian cancer markers (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-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 biomarkers can be used together for determining a prognosis for survival for a colon cancer patient.

**[0085]** 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 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 (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 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.

**[0086]** 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,  $\beta$ -catenin, MIB-1 and a variety of other general and tumor-specific biomarkers, such as commercially available 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 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.

**[0087]** The invention also provides a method for monitoring the effectiveness of a course of treatment for a patient with colon cancer. The method involves (a) determining the level of TUCAN in a neoplastic cell-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 biomarker level after treatment indicates the effectiveness of the treatment wherein a decrease in the level of TUCAN after treatment indicates that treatment is effective.

**[0088]** 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 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 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 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  $\beta$ -catenin, cIAP2 or TUCAN in a patient sample after treatment indicates that treatment is effective because low levels of  $\beta$ -catenin, cIAP2 or TUCAN correlate with a lower incidence of colon cancer recurrence.

**[0089]** Patients having cancer can be classified according to whether a high level of a particular 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 lower likelihood of disease-free or overall survival with respect to the group to which the patient was not assigned.

**[0090]** Therefore, the invention also provides a method of determining a prognosis for survival for a colon cancer 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 levels of TUCAN lower than the reference level is classified as having an increased likelihood of survival compared to the second group of patients having levels of TUCAN higher than the reference level.

**[0091]** A level of TUCAN higher than the reference level, or overproduction of TUCAN, correlates with patients having an increased risk of tumor recurrence or spread. Thus, patients belonging to a first group having levels of TUCAN higher than the reference level are classified as having an increased risk of tumor recurrence or spread compared to a second group of patients having levels TUCAN lower than the reference level. Patients belonging to a first group having levels of TUCAN lower than the reference level are classified as having increased likelihood of survival compared to a second group of patients having levels of TUCAN higher than the reference level.

**[0092]** The method of determining a prognosis for survival for a colon cancer patient can be practiced using one or more additional biomarkers. A variety of biomarkers, 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-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 levels of TUCAN and levels of cIAP2 lower than the reference level is classified as having increased likelihood of survival compared to the second group of patients having levels of TUCAN and levels of cIAP2 higher than the reference level.

**[0093]** 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 first group of patients having levels of TUCAN lower than the reference level and levels of any of Apaf1, Smac or Bcl-2 higher than the reference level is classified as having increased likelihood of survival compared to the second group of patients having levels of TUCAN higher than the reference level and levels of any of Apaf1, Smac or Bcl-2 lower than the reference level.

**[0094]** 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 overall survival for the patient is assessed based on the likelihood of disease-free or overall survival for patients in that group.

**[0095]** For example, a neoplastic cell containing sample from a colon cancer patient can be determined to have 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 levels of Apaf1, Bcl-2 or Smac higher than the reference level. Because it has been discovered that there is an increased likelihood of disease-free or overall survival for the group of patients expressing levels of Apaf1, Bcl-2 or Smac higher than the reference level in cancer cells (relative to those expressing levels of Apaf1, Bcl-2 or Smac lower than the reference level in cancer cells), the specific cancer patient would be considered to have an increased likelihood of disease free or overall survival.

**[0096]** Conversely, a neoplastic cell containing sample from a colon cancer patient can be determined to have high levels of cIAP2,  $\beta$ -catenin or TUCAN relative to a reference level. This patient would then be classified into a group of patients having levels of cIAP2,  $\beta$ -catenin or TUCAN higher than the reference level. Because it has been discovered that there is a decreased likelihood of disease-free or overall survival for the group of patients expressing levels of cIAP2,  $\beta$ -catenin or TUCAN higher than the reference level in cancer cells (relative to those expressing levels of cIAP2,  $\beta$ -catenin or TUCAN lower than the reference level in cancer cells), the specific cancer patient would be considered to have an decreased likelihood of disease free or overall survival.

**[0097]** The methods of the invention are applicable to determining the susceptibility of an individual for developing colon cancer. The methods could be applicable to a variety of other cancers, including gastrointestinal, lung, prostate, breast, ovarian, skin, blood and kidney cancers. Colon cancers develop from premalignant precursor lesions known as adenomatous colon polyps. Multiple epidemiological studies have 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 is practiced as described herein for colon neoplastic conditions, such as adenomatous colon polyps, for example, using an appropriately obtained biopsy sample.

**[0098]** The methods of the invention for determining a prognosis for survival for a colon cancer patient are applicable to patients at any stage of tumor progression, and might 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 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 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.

**[0099]** 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. 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 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 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 has spread to distant organs such as the liver, lung, peritoneum or ovary.

**[0100]** 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 lymph nodes local to the organ of the primary tumor. Typically, early stages will be considered to be stages I and II.

**[0101]** The predictive value of the method of the invention will be particularly effective in the case of patients in the early stages of colon 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 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 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.

5 [0102] A stage of cancer progression can be correlated 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.

10 [0103] 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 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 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 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.

15 [0104] 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 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.

20 [0105] Whole tissue obtained from a biopsy or surgery 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 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 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 the samples are similarly well known in the art.

25 [0106] 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 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 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 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.

30 [0107] The tissue or whole cell tumor cell sample 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. 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 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 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 skilled in the art and can accordingly be used in the methods of the invention.

35 [0108] 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 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, 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.

5 [0109] 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 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 from an individual having, or suspected of having a colon neoplastic condition or colon cancer.

10 [0110] Fluid samples, which can be measured for biomarker levels, include, for example, blood, serum, lymph, urine and stool. Other bodily fluids are known to 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 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.

15 [0111] 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 can be measured by a variety of methods known in the art.

20 [0112] 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 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 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 samples simultaneously, such as in a clinical setting, then a multi-sample 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 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.

25 [0113] Nucleic acid probes can be produced 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 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., *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).

30 [0114] Nucleic acid probes useful for detecting a 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 biomarker in a particular sample type.

35 [0115] 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.

40 [0116] 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,  $\beta$ -catenin or another biomarker.

**[0117]** The invention relates to the discovery that amounts of particular biomarkers higher or lower than the reference level, including cIAP2, TUCAN, Apaf1, Bcl-2,  $\beta$ -catenin and Smac are predictive of survival of patients having colon 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.

**[0118]** A method for treating or reducing the progression of a neoplastic condition such as cancer by reducing neoplastic cell proliferation could be provided. The method could involve administering a nucleic acid encoding Apaf1, Bcl-2 or Smac into a neoplastic cell and expressing the Apaf1, Bcl-2 or Smac polypeptide in an amount effective to reduce neoplastic cell proliferation. Alternatively, the method of reducing neoplastic cell proliferation could involve contacting a neoplastic cell with an effective amount of an agent that, under sufficient conditions, increases the amount of Apaf1, Bcl-2 or Smac in the cell.

**[0119]** Such an agent can increase the amount of a biomarker directly or indirectly, for example, by 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 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 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, 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 the length of time that gene expression remains active.

**[0120]** 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 the method of reducing neoplastic cell proliferation could involve 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.

**[0121]** 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,  $\beta$ -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.

**[0122]** Ribozymes, enzymatic RNA molecules, can also be used to catalyze the specific cleavage of a biomarker mRNA, such as cIAP2,  $\beta$ -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 can be prepared by any method known in the art for the synthesis of nucleic acid molecules.

**[0123]** RNA interference (RNAi) can also be used to modulate the amount of a biomarker mRNA, such as cIAP2,  $\beta$ -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.

5 **[0124]** 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).

15 **[0125]** 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.

20 **[0126]** 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.

35 **[0127]** 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. 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, Bcl-2 or Smac polypeptide can be operatively linked to a cell specific promoter.

40 **[0128]** Any of a variety of inducible promoters or enhancers can also be included in a nucleic acid or vector to allow control of expression of 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 & 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. 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 temperature changes.

50 **[0129]** 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 an antiprogestin 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 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 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 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.

[0130] The following examples are intended to illustrate but not limit the present invention.

## EXAMPLE I

### Generation of Antibodies for Immunodetection of IAPs and Apaf1

[0131] This example shows preparation and characterization of antibodies useful for detecting IAPs and Apaf1.

[0132] Antisera were raised against recombinant proteins and synthetic peptides for immunodetection of 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 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. 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 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, 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.

[0133] 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.

[0134] 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 (NH<sub>2</sub> -CGPKYVVPVESSLGKEKGLE -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 injection 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

[0135] This example shows immunohistochemical analysis of IAPs and other biomarkers in a tissue microarray representing tissue samples obtained from 102 individuals.

[0136] 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 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 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 patients after relapse. Clinical data represent a median follow up of 60 months.

**[0137]** 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  $\mu$ m) were applied to 3-aminopropyltriethoxysilane (APES)-coated slides (Sigma).

**[0138]** 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,  $\beta$ -Catenin, MIB-1 and p53. Dewaxed tissue sections were 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  $\beta$ -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  $\mu$ 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).

**[0139]** 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).

**[0140]** 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.

**[0141]** 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 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 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 examined, evidence of tumor-associated upregulation of expression was observed in a portion of the cancer specimens evaluated.

**[0142]** Elevated levels of cIAP2, Survivin, and  $\beta$ -Catenin correlated with high Ki-67 labeling index ( $p = 0.006$ ,  $p = 0.005$ , 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 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 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 concentration of p53, which in 80% is related to p53 point missense mutation correlated with increased expression of Bcl-XL ( $p < .0001$ ).

### EXAMPLE III

#### Immunoblot Analysis of IAPs and Apaf1 in Colon Carcinoma

**[0143]** This example shows immunoblot analysis of IAPs, Apaf1 and other apoptosis-regulators in five frozen colon cancer specimens.

**[0144]** To corroborate the immunohistochemistry data, 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 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).

**[0145]** 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 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 Na<sub>3</sub>VO<sub>4</sub>, 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 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 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 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 intensity of bands, using Image-pro Plus software.

**[0146]** 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 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.

**[0147]** The immunoblotting results confirmed the 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**

**[0148]** 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.

**[0149]** 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 (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 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). 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 $\pm$ SE Median	Mean $\pm$ SE Median	Mean $\pm$ SE Median	Mean $\pm$ SE Median	Mean $\pm$ SE Median
<b>A</b>	168 $\pm$ 9 160	152 $\pm$ 10 160	166 $\pm$ 9 190	81 $\pm$ 9 70	132 $\pm$ 8 140
<b>R</b>	176 $\pm$ 22 170	125 $\pm$ 25 95	169 $\pm$ 23 180	162 $\pm$ 22 160	46 $\pm$ 20 50
<b>D</b>	177 $\pm$ 13 180	135 $\pm$ 13 120	174 $\pm$ 13 180	146 $\pm$ 13 130	77 $\pm$ 12 70
p-values A vs R+D	0.5	0.2	0.7	<.0001	<.0001

**[0150]** 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 epithelium and colon cancers shown in Figure 2 were used to set logical cut-offs for dichotomization of data. Immunoscoring for normal and malignant colon epithelium were depicted in a graphic form in Figure 2. Based on comparisons with normal colonic epithelium, cutoffs for 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 cut-offs for Bcl-2, Bid, AIF, Smac, and  $\beta$ -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 high levels of MIB.

**[0151]** 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 correlations were observed in this cohort between longer disease-free survival (DFS) and low expression of cIAP2 ( $p = 0.0002$ ), TUCAN ( $p = 0.0004$ ),  $\beta$ -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 ( $p = 0.03$ ) (Figure 4A-4P). 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 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 patients remaining disease-free compared to only 28/62 (45%) of those with low Apaf1 expression.

**[0152]** 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 4Q-4F-1). Among 50 patients expressing low 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 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).

**[0153]** 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 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 effect of Bcl-2 on clinical outcome may be linked to its cell cycle-inhibitory role.

**[0154]** Figure 4 shows correlations of biomarkers immunostaining data with disease-free (A-P) survival and overall survival (Q-F-1) for colon carcinoma patients. All 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.

**[0155]** In summary, 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. At the same time point, 49% of patients with high expression of TUCAN 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. 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. 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 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

**[0156]** This example shows combined analysis of cIAP2 and Apaf1 expression data.

**[0157]** 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 favorable variables (low cIAP2 and high Apaf1) were compared with all other patients in this cohort.

**[0158]** 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).

**[0159]** 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 5C). 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 ( $p = .004$ ) (Figure 5D). These results are in agreement with an outcome of the LERS data analysis.

## EXAMPLE VI

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

**[0160]** This example shows that multivariate analysis confirms that cIAP2, Apaf1, TUCAN and Bcl-2 are independent prognostic indicators of survival in early-stage colon cancer.

**[0161]** 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 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 data.

**[0162]** 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 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% ( $p = 0.00006$ ).

**[0163]** 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	coefficient	HR (95% CI)	p
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
B. OS			
BIOMARKER	coefficient	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
Bcl-2	-1.69	0.18 (0.08 - 0.43)	0.00006

## EXAMPLE VII

Expression of TUCAN in Multiple Cancer Cell Lines

5 [0164] This example shows that TUCAN is expressed in several tumor cell lines.

[0165] 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 are shown in Table 4, below:

10 Table 4: NCI panel of 60 human tumor cell lines

Cell Line Name	Cell Type
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 EK VX	Non-Small Cell Lung
HOP-62	Non-Small Cell Lung
HOP-92	Non-Small Cell Lung
NCI-H226	Non-Small Cell Lung
25 NCI-H23	Non-Small Cell Lung
NCI-H322M	Non-Small Cell Lung
NCI-H460	Non-Small Cell Lung
NCI-H522	Non-Small Cell Lung
COLO 205	Colon
30 HCC-2998	Colon
HCT-116	Colon
HCT-15	Colon
HT29	Colon
35 KM12	Colon
SW-620	Colon
SF-268	CNS
SF-295	CNS
SF-539	CNS
40 SNB-19	CNS
SNB-75	CNS
U251	CNS
LOX IMVI	Melanoma
45 MALME-3M	Melanoma
M14	Melanoma
SK-MEL-2	Melanoma
SK-MEL-28	Melanoma
SK-MEL-5	Melanoma
50 UACC-257	Melanoma
UACC-62	Melanoma
IGR-OV1	Ovarian
OVCAR-3	Ovarian
OVCAR-4	Ovarian
55 OVCAR-5	Ovarian
OVCAR-8	Ovarian
SK-OV-3	Ovarian

(continued)

	Cell Line Name	Cell Type
	786-0	Renal
5	A498	Renal
	ACHN	Renal
	CAKI-1	Renal
	RXF 393	Renal
10	SN12C	Renal
	TK-10	Renal
	UO-31	Renal
	PC-3	Prostate
	DU-145	Prostate
15	MCF7	Breast
	NCI/ADR-RES	Breast
	MDA-MB-231/ATCC	Breast
	HS 578T	Breast
	MDA-MB-435	Breast
20	MDA-N	Breast
	BT-549	Breast
	T-47D	Breast

25 **[0166]** 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, 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 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 cancer cells (Figure 6A).

30 **[0167]** 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 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.

35 **[0168]** In summary, TUCAN is expressed in a variety of tumor cell lines, including cancer cells obtained from human breast, ovarian, lung, CNS, leukemia, kidney, prostate, skin and colon tumors.

#### 40 **EXAMPLE VIII**

##### **Elevated TUCAN Expression in Colon Cancers Correlates with Reduced Patient Survival**

45 **[0169]** This example shows that TUCAN expression is elevated in colon cancers and that TUCAN elevation correlates with reduced colon cancer patient survival.

50 **[0170]** 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 (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 immunointensity due to technical artifacts (Figure 7).

55 **[0171]** 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 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.

**[0172]** 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).

**[0173]** 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.

**[0174]** 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.

**[0175]** 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.

**[0176]** 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
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
Dead from disease	31	90 ± 4	95	2.5 ± 0.1	3	224 ± 12	240
p-values	102	p < .0001		p < .0001		p < .0001	

**[0177]** 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**

**[0178]** 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.

**[0179]** 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 substitute 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).

**[0180]** 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) ( $\Delta$ CARD). Pro-Caspase-9 co-immunoprecipitated with full-length TUCAN and the CARD only fragment but not the  $\Delta$ 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  $\Delta$ CARD proteins. Full-length TUCAN interacted with full-length TUCAN and the CARD-only fragment but not the  $\Delta$ CARD fragment (Figure 8C). Thus, the CARD domain of TUCAN is necessary and sufficient for self-association.

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**[0181]**

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<120> Methods For Determining the Prognosis For Cancer Patients Using Tucan

<130> FP-LJ 5246

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	Leu	Val	Ala	His	Phe	Lys	Asn	Glu	Gly	Met	Val	Leu	Glu	His	Pro	Ala
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	Ile	Asp	Asp	Glu	Glu	Asp	Arg	Phe	His	Gly	Val	Arg	Leu	Gln	Thr	Ser
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 Glu Asn Glu Lys Glu Leu Val Glu Gln Glu Lys Thr Arg Gln Ser Lys  
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 Gly Gln Asp Met Leu Ala Ser Phe Leu Arg Thr Asn Arg Gln Ala Ala  
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 Pro Glu Ile Arg Lys Pro Glu Val Leu Arg Pro Glu Thr Pro Arg Pro  
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 Leu Arg Gly Asn Ala Asp Leu Ala Tyr Ile Leu Ser Met Glu Pro Cys  
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 45 Gly His Cys Leu Ile Ile Asn Asn Val Asn Phe Cys Arg Glu Ser Gly  
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 180 185 190  
 Arg Phe Ser Ser Pro His Phe Met Val Glu Val Lys Gly Asp Leu Thr  
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 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
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Asp Glu Ser Pro Gly Ser Asn Pro Glu Pro Asp Ala Thr Pro Phe Gln
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Thr Pro Ser Asp Ile Phe Val Ser Tyr Ser Thr Phe Pro Gly Phe Val
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Ser Trp Arg Asp Pro Lys Ser Gly Ser Trp Tyr Val Glu Thr Leu Asp
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	Glu	His	Leu	Arg	His	Phe	Pro	Lys	Cys	Pro	Phe	Ile	Glu	Asn	Gln	Leu	
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65	caa	gac	act	tca	aga	tac	aca	ggt	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	
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35 caa gct tta gga ctt cac ctg aca gat cca agt caa cgt ctt gtt cag 1337  
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40 aac tgt ctt tgg act ctc agg aat ctt tca gat gct gca act aaa cag 1385  
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 Ile Thr Glu Pro Ala Ile Cys Ala Leu Arg His Leu Thr Ser Arg His  
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	Val	Lys	Met	Leu	Gly	Ser	Pro	Val	Asp	Ser	Val	Leu	Phe	Tyr	Ala	Ile
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Leu His Ile Leu Ala Arg Asp Val His Asn Arg Ile Val Ile Arg Gly  
 580 585 590  
 Leu Asn Thr Ile Pro Leu Phe Val Gln Leu Leu Tyr Ser Pro Ile Glu  
 595 600 605  
 Asn Ile Gln Arg Val Ala Ala Gly Val Leu Cys Glu Leu Ala Gln Asp  
 610 615 620  
 Lys Glu Ala Ala Glu Ala Ile Glu Ala Glu Gly Ala Thr Ala Pro Leu  
 625 630 635 640  
 Thr Glu Leu Leu His Ser Arg Asn Glu Gly Val Ala Thr Tyr Ala Ala  
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 His Glu Met Gly Gly His His Pro Gly Ala Asp Tyr Pro Val Asp Gly  
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55 aat tgt ttg ctt caa cat aga gaa gct ctg gaa aag gac atc aag aca 643  
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10	gaa gag gaa aaa gta aga aat gag ccc act caa cag caa aga gca gct Glu Glu Glu Lys Val Arg Asn Glu Pro Thr Gln Gln Gln Arg Ala Ala	40	45	50	739	
15	atg ctg att aaa atg ata ctt aaa aaa gat aat gat tcc tac gta tca Met Leu Ile Lys Met Ile Leu Lys Lys Asp Asn Asp Ser Tyr Val Ser	55	60	65	70	787
20	ttc tac aat gct cta cta cat gaa gga tat aaa gat ctt gct gcc ctt Phe Tyr Asn Ala Leu Leu His Glu Gly Tyr Lys Asp Leu Ala Ala Leu	75	80	85	835	
25	ctc cat gat ggc att cct gtt gtc tct tct tcc agt gta agg aca gtc Leu His Asp Gly Ile Pro Val Val Ser Ser Ser Ser Val Arg Thr Val	90	95	100	883	
30	ctg tgt gaa ggt gga gta cca cag agg cca gtt gtt ttt gtc aca agg Leu Cys Glu Gly Gly Val Pro Gln Arg Pro Val Val Phe Val Thr Arg	105	110	115	931	
35	aag aag ctg gtg aat gca att cag cag aag ctc tcc aaa ttg aaa ggt Lys Lys Leu Val Asn Ala Ile Gln Gln Lys Leu Ser Lys Leu Lys Gly	120	125	130	979	
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45	gta tta gct gca gaa gct gtt aga gat cat tcc ctt tta gaa ggt tgt Val Leu Ala Ala Glu Ala Val Arg Asp His Ser Leu Leu Glu Gly Cys	155	160	165	1075	
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60	gag agt ttt tcc cag agg ctt cca ctt aat att gaa gag gct aaa gac Glu Ser Phe Ser Gln Arg Leu Pro Leu Asn Ile Glu Glu Ala Lys Asp	200	205	210	1219	
65	cgT ctc cgc att ctg atg ctt cgc aaa cac cca agg tct ctc ttg atc Arg Leu Arg Ile Leu Met Leu Arg Lys His Pro Arg Ser Leu Leu Ile	215	220	225	230	1267

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 Cys Gln Ile Leu Leu Thr Thr Arg Asp Lys Ser Val Thr Asp Ser Val  
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 505 510 515

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 520 525 530

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 gag ttt tta tct tta aat gga cac ctt ctt gga cga cag cca ttt cct 2227  
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 555 560 565

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 Gln Gln Ala Lys Leu Gln Ala Lys Gln Glu Val Asp Asn Gly Met Leu  
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	Glu	Leu	Val	His	Thr	Tyr	Asp	Glu	His	Ser	Glu	Gln	Val	Asn	Cys	Cys	
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	His	Phe	Thr	Asn	Ser	Ser	His	His	Leu	Leu	Leu	Ala	Thr	Gly	Ser	Ser	
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20	gac	tgc	ttc	ctc	aaa	ctt	tgg	gat	ttg	aat	caa	aaa	gaa	tgt	cga	aat	2755
	Asp	Cys	Phe	Leu	Lys	Leu	Trp	Asp	Leu	Asn	Gln	Lys	Glu	Cys	Arg	Asn	
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	Asp	Asp	Lys	Leu	Leu	Ala	Ser	Cys	Ser	Ala	Asp	Gly	Thr	Leu	Lys	Leu	
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	Trp	Asp	Ala	Thr	Ser	Ala	Asn	Glu	Arg	Lys	Ser	Ile	Asn	Val	Lys	Gln	
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**Claims**

- 55 1. A method for determining a prognosis for survival for a colon cancer patient, comprising:
- (a) measuring a level of tumor up-regulated CARD-containing antagonist of caspase nine (TUCAN) in a neo-plastic 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 level of TUCAN lower than the reference level in said sample correlates with increased survival of said patient.

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

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

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4. A method of claim 1, further comprising:

(a) measuring levels of one or more biomarkers selected from the group consisting of apoptosis inhibitor 2 (clAP2), apoptotic protease activating factor 1 (Apaf1), B-cell Lymphoma protein 2 (Bcl-2) and second mitochondria-derived activator of caspase (Smac) in the neoplastic cell-containing sample from said cancer patient, and

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(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 or more selected biomarkers, wherein a level of TUCAN lower than the reference level and a level of any of Apaf1, Bcl-2 or Smac higher than the reference levels, or a level of TUCAN and a level of clAP2 lower than the reference levels, in said sample correlate with increased survival of said patient.

20

5. The method of claim 4, wherein clAP2 is a selected biomarker.

6. The method of claim 4 or 5, wherein Apaf1 is a selected biomarker.

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7. The method of claim 4 to 6, wherein Bcl-2 is a selected biomarker.

8. The method of claim 4 to 7, wherein Smac is a selected biomarker.

9. The method of claims 4 to 8, wherein a level of TUCAN or a biomarker polypeptide is measured.

30

10. The method of claim 9, wherein a level of TUCAN or a biomarker polypeptide is measured using an antibody specifically reactive with TUCAN or the biomarker polypeptide.

11. The method of claims 4 to 8, wherein a level of TUCAN or a biomarker nucleic acid is measured.

35

12. The method of claim 4, wherein the levels of TUCAN and one or more biomarkers are used to determine if said patient is at risk for relapse.

13. The method of claim 4, wherein the levels of TUCAN and one or more biomarkers are used to determine a proper course of treatment for said patient.

40

14. The method of claim 4, further comprising selecting two or more biomarkers from the group consisting of clAP2, Apaf1, Bcl-2 and Smac.

45

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

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

50

(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 indicates the effectiveness of said treatment, wherein a decrease in the level of TUCAN after treatment indicates that treatment is effective.

16. A method of claim 1, comprising:

55

(c) classifying said patient as belonging to either a first or second group of patients, wherein said first group of patients having levels of TUCAN lower than the reference level is classified as having an increased likelihood of survival compared to said second group of patients having levels of TUCAN higher than the reference level.

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17. The method of claims 1 to 14 and 16, wherein said survival is overall survival.

18. The method of claims 1 to 14 and 16, wherein said survival is disease-free survival.

5 19. The method of claims 1 to 18, wherein said sample is colon tumor tissue.

20. The method of claims 1 to 18, wherein said sample is a fluid selected from the group consisting of blood, serum, urine, semen and stool.

10 21. The method of claims 1 to 3, 15 and 16, wherein a level of a TUCAN polypeptide is measured.

22. The method of claims 1 to 3, 15 and 16, wherein a level of a TUCAN polypeptide is measured using an antibody specifically reactive with TUCAN polypeptide.

15 23. The method of claims 1 to 3, 15 and 16, wherein a level of TUCAN nucleic acid is measured.

24. The method of claims 1 to 23, wherein said patient has an early stage of cancer.

25. The method of claim 16, further comprising:

20 (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 levels of TUCAN and levels of cIAP2 lower than the reference levels is classified as having increased likelihood of survival compared to said second group of patients having levels of TUCAN and levels  
25 of cIAP2 higher than the reference levels.

26. The method of claim 16, further comprising:

30 (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 either a first or second group of patient, wherein said first group of patients having levels of TUCAN lower than the reference level and levels of any of Apaf1, Smac or Bcl-2 higher than the reference levels is classified as having increased likelihood of survival compared to said second group of patients having levels of TUCAN higher than the reference level and levels of any of Apaf1, Smac or Bcl-2  
35 lower than the reference levels.

27. The method of claim 16, further comprising determining a level of a biomarker selected from the group consisting of cIAP2, Apaf1, Smac and Bcl-2 in said 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  
40 of the selected biomarker and TUCAN after treatment to indicate the effectiveness of said treatment.

28. The method of claims 1 to 27, wherein the patient has stage II colon carcinoma.

### 45 Patentansprüche

1. Verfahren zur Bestimmung der Prognose des Überlebens für einen Colonkrebs-Patienten, Schritte umfassend, bei denen man:

50 (a) eine Konzentration von Tumor up-regulated CARD-containing antagonist of caspase nine (TUCAN) in einer neoplastische Zellen enthaltenden Probe des Krebspatienten misst und  
(b) die Konzentration von TUCAN in der Probe mit einer Referenzkonzentration von TUCAN vergleicht,

wobei eine Konzentration von TUCAN in der Probe niedriger als die Referenzkonzentration mit einem erhöhten  
55 Überleben des Patienten korreliert.

2. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Konzentration von TUCAN verwendet wird, um festzustellen, ob für den Patienten das Risiko eines Rückfalls besteht.

3. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Konzentration von TUCAN verwendet wird, um eine richtige Art der Behandlung für den Patienten festzulegen.

4. Verfahren nach Anspruch 1, ferner Schritte umfassend, bei denen man

(a) die Konzentration von einem oder mehreren Biomarkern, ausgewählt aus der Gruppe bestehend aus Apoptosis Inhibitor 2 (cIAP2), Apoptotic protease activating factor 1 (Apaf1), B-cell lymphoma protein 2 (Bcl-2) und Second mitochondria derived activator of caspase (Smac) in der neoplastische Zellen enthaltene Probe des Krebspatienten misst und

(b) die Konzentration von TUCAN und dem einen oder den mehreren ausgewählten Biomarkern in der Probe mit einer Referenzkonzentration von TUCAN und dem einen oder den mehreren ausgewählten Biomarkern vergleicht, wobei eine Konzentration von TUCAN in der Probe niedriger als die Referenzkonzentration und eine Konzentration von einem aus Apaf1, Bcl-2 oder Smac höher als die Referenzkonzentrationen oder eine Konzentration von TUCAN und eine Konzentration von cIAP2 niedriger als die Referenzkonzentrationen mit einem erhöhten Überleben des Patienten korrelieren.

5. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** cIAP2 ein ausgewählter Biomarker ist.

6. Verfahren nach Anspruch 4 oder 5, **dadurch gekennzeichnet, dass** Apaf1 ein ausgewählter Biomarker ist.

7. Verfahren nach Anspruch 4 bis 6, **dadurch gekennzeichnet, dass** Bcl-2 ein ausgewählter Biomarker ist.

8. Verfahren nach Anspruch 4 bis 7, **dadurch gekennzeichnet, dass** Smac ein ausgewählter Biomarker ist.

9. Verfahren nach den Ansprüchen 4 bis 8, **dadurch gekennzeichnet, dass** eine Konzentration von TUCAN- oder einem Biomarker-Polypeptid gemessen wird.

10. Verfahren nach Anspruch 9, **dadurch gekennzeichnet, dass** eine Konzentration von TUCAN- oder einem Biomarker-Polypeptid unter Verwendung eines spezifisch mit dem TUCAN- oder dem Biomarker-Polypeptid reaktiven Antikörpers gemessen wird.

11. Verfahren nach den Ansprüchen 4 bis 8, **dadurch gekennzeichnet, dass** eine Konzentration einer TUCAN- oder einer Biomarker-Nukleinsäure gemessen wird.

12. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** die Konzentrationen von TUCAN und dem einen oder den mehreren Biomarkern verwendet werden, um festzustellen, ob für den Patienten das Risiko eines Rückfalls besteht.

13. Verfahren nach Anspruch 4, **dadurch gekennzeichnet, dass** die Konzentrationen von TUCAN und dem einen oder den mehreren Biomarkern verwendet werden, um eine richtige Art der Behandlung für den Patienten festzulegen.

14. Verfahren nach Anspruch 4, ferner umfassend, dass man 2 oder mehr Biomarker aus der Gruppe bestehend aus cIAP2, Apaf1, Bcl-2 und Smac auswählt.

15. Verfahren zur Überwachung der Effektivität einer Art der Behandlung für einen Patienten mit Colonkrebs, Schritte umfassend, bei denen man:

(a) eine Konzentration von TUCAN in einer neoplastische Zellen enthaltenden Probe von einem Krebspatienten vor der Behandlung bestimmt und

(b) die Konzentration von TUCAN in einer neoplastische Zellen enthaltenden Probe des Patienten nach der Behandlung bestimmt, wobei der Vergleich der Konzentration von TUCAN vor der Behandlung mit der Konzentration von TUCAN nach der Behandlung auf die Effektivität der Behandlung hinweist, wobei eine Abnahme der Konzentration von TUCAN nach der Behandlung darauf hinweist, dass die Behandlung effektiv ist.

16. Verfahren nach Anspruch 1, ferner umfassend, dass man:

(c) den Patienten als zugehörig entweder zu einer ersten oder einer zweiten Gruppe von Patienten klassifiziert,

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wobei die erste Gruppe von Patienten, welche Konzentrationen von TUCAN geringer als die Referenzkonzentration aufweist, so klassifiziert wird, dass sie im Vergleich zu der zweiten Gruppe an Patienten, welche eine Konzentration von TUCAN höher als die Referenzkonzentration aufweist, eine erhöhte Wahrscheinlichkeit des Überlebens aufweist.

- 5
17. Verfahren nach den Ansprüchen 1 bis 14 und 16, **dadurch gekennzeichnet, dass** das Überleben Gesamtüberleben ist.
- 10
18. Verfahren nach den Ansprüchen 1 bis 14 und 16, **dadurch gekennzeichnet, dass** das Überleben krankheitsfreies Überleben ist.
- 15
19. Verfahren nach den Ansprüchen 1 bis 18, **dadurch gekennzeichnet, dass** die Probe Colon-Tumorgewebe ist.
20. Verfahren nach den Ansprüchen 1 bis 18, **dadurch gekennzeichnet, dass** die Probe eine Flüssigkeit ausgewählt aus der Gruppe bestehend aus Blut, Serum, Urin, Samen und Stuhl ist.
- 20
21. Verfahren nach den Ansprüchen 1 bis 3, 15 und 16, **dadurch gekennzeichnet, dass** eine Konzentration eines TUCAN-Polypeptids gemessen wird.
- 25
22. Verfahren nach den Ansprüchen 1 bis 3, 15 und 16, **dadurch gekennzeichnet, dass** eine Konzentration eines TUCAN-Polypeptids unter Verwendung eines spezifisch mit TUCAN-Polypeptid reaktiven Antikörpers gemessen wird.
- 25
23. Verfahren nach den Ansprüchen 1 bis 3, 15 und 16, **dadurch gekennzeichnet, dass** eine Konzentration einer TUCAN-Nukleinsäure gemessen wird.
- 30
24. Verfahren nach den Ansprüchen 1 bis 23, **dadurch gekennzeichnet, dass** der Patient ein frühes Krebsstadium hat.
- 30
25. Verfahren nach Anspruch 16, ferner Schritte umfassend, bei denen man:
- (a) eine Konzentration von cIAP2 in der neoplastische Zellen enthaltenden Probe des Krebspatienten bestimmt und
- (b) den Patienten als zugehörig entweder zu einer ersten oder einer zweiten Patientengruppe klassifiziert, wobei die erste Gruppe von Patienten, welche Konzentrationen von TUCAN und Konzentrationen von cIAP2 geringer als die Referenzkonzentrationen aufweist, so klassifiziert wird, dass sie im Vergleich zu der zweiten Gruppe an Patienten, welche Konzentrationen von TUCAN und Konzentrationen von cIAP2 höher als die Referenzkonzentrationen aufweist, eine erhöhte Wahrscheinlichkeit des Überlebens aufweist.
- 35
- 40
26. Verfahren nach Anspruch 16, ferner Schritte umfassend, bei denen man:
- (a) eine Konzentration eines Biomarkers, ausgewählt aus der Gruppe bestehend aus Apaf1, Smac und Bcl-2 in der neoplastische Zellen enthaltenden Probe des Krebspatienten bestimmt und
- (b) den Patienten als zugehörig entweder zu einer ersten oder einer zweiten Patientengruppe klassifiziert, wobei die erste Gruppe von Patienten, welche Konzentrationen von TUCAN geringer als die Referenzkonzentration aufweist und Konzentrationen von einem von Apaf1, Smac oder Bcl-2 höher als die Referenzkonzentrationen aufweist, so klassifiziert wird, dass sie im Vergleich zu der zweiten Gruppe an Patienten, welche Konzentrationen von TUCAN höher als die Referenzkonzentration und Konzentrationen von einem von Apaf1, Smac oder Bcl-2 geringer als die Referenzkonzentrationen aufweist, eine erhöhte Wahrscheinlichkeit des Überlebens aufweist.
- 45
- 50
27. Verfahren nach Anspruch 16, ferner Schritte umfassend, bei denen man eine Konzentration eines Biomarkers, ausgewählt aus der Gruppe bestehend aus cIAP2, Apaf1, Smac und Bcl-2 in der neoplastische Zellen enthaltenden Probe des Krebspatienten vor und nach Behandlung bestimmt, wobei die Konzentrationen der ausgewählten Biomarker und von TUCAN vor der Behandlung mit den Konzentrationen der ausgewählten Biomarker und von TUCAN nach der Behandlung verglichen werden, um auf die Effektivität der Behandlung hinzuweisen.
- 55
28. Verfahren nach den Ansprüchen 1 bis 27, **dadurch gekennzeichnet, dass** der Patient ein Colonkarzinom in Stadium II hat.

**Revendications**

1. Procédé pour la détermination d'un pronostic pour la survie d'un patient ayant un cancer du côlon, comprenant :
  - 5 (a) la mesure d'un niveau de TUCAN, "tumor up-regulated CARD-containing antagonist of caspase nine" (antagoniste de la caspase-9, contenant un domaine CARD (dont l'expression est régulée positivement dans les tumeurs)), dans un échantillon contenant des cellules néoplasiques provenant dudit patient ayant un cancer, et
  - (b) la comparaison du niveau de TUCAN dans ledit échantillon à un niveau de référence de TUCAN,
- 10 dans lequel un niveau de TUCAN inférieur au niveau de référence dans ledit échantillon est en corrélation avec une survie prolongée dudit patient.
2. Procédé selon la revendication 1, dans lequel ledit niveau de TUCAN est utilisé pour déterminer si ledit patient est à risque de récurrence.
- 15 3. Procédé selon la revendication 1, dans lequel ledit niveau de TUCAN est utilisé pour déterminer une évolution appropriée du traitement pour ledit patient.
- 20 4. Procédé selon la revendication 1, comprenant en outre:
  - (a) la mesure de niveaux de un ou plusieurs biomarqueurs choisis dans le groupe constitué d'un inhibiteur 2 de l'apoptose (cIAP2), d'un facteur 1 d'activation des protéases apoptotiques (Apaf1), d'une protéine 2 d'un lymphome malin à cellules B (Bcl-2) et d'un second activateur de caspase dérivé des mitochondries (Smac) dans l'échantillon contenant des cellules néoplasiques provenant dudit patient ayant un cancer et,
  - 25 (b) la comparaison du niveau de TUCAN et d'un ou plusieurs biomarqueurs choisis dans ledit échantillon à un niveau de référence de TUCAN et dudit un ou plusieurs biomarqueurs choisis, dans lequel un niveau de TUCAN inférieur au niveau de référence et un niveau de l'un quelconque de Apaf1, Bcl-2 ou Smac supérieur aux niveaux de référence, ou un niveau de TUCAN et un niveau de cIAP2 inférieur aux niveaux de référence, dans ledit échantillon, sont en corrélation avec une survie prolongée dudit patient.
- 30 5. Procédé selon la revendication 4, dans lequel cIAP2 est un biomarqueur choisi.
6. Procédé selon la revendication 4 ou 5, dans lequel Apaf1 est un biomarqueur choisi.
- 35 7. Procédé selon les revendications 4 à 6, dans lequel Bcl-2 est un biomarqueur choisi.
8. Procédé selon les revendications 4 à 7, dans lequel Smac est un biomarqueur choisi.
9. Procédé selon les revendications 4 à 8, dans lequel un niveau de TUCAN ou d'un polypeptide biomarqueur est mesuré.
- 40 10. Procédé selon la revendication 9, dans lequel un niveau de TUCAN ou d'un polypeptide biomarqueur est mesuré en utilisant un anticorps spécifiquement réactif vis-à-vis de TUCAN ou du polypeptide biomarqueur.
- 45 11. Procédé selon les revendications 4 à 8, dans lequel un niveau de TUCAN ou d'un acide nucléique de biomarqueur est mesuré.
12. Procédé selon la revendication 4, dans lequel les niveaux de TUCAN et d'un ou plusieurs biomarqueurs sont utilisés pour déterminer si ledit patient présente un risque de récurrence.
- 50 13. Procédé selon la revendication 4, dans lequel les niveaux de TUCAN et d'un ou plusieurs biomarqueurs sont utilisés pour déterminer une évolution appropriée du traitement pour ledit patient.
- 55 14. Procédé selon la revendication 4, comprenant en outre le choix de deux ou plusieurs biomarqueurs dans le groupe constitué de cIAP2, Apaf1, Bcl-2 et Smac.
15. Procédé pour la surveillance de l'efficacité d'un traitement pour un patient ayant un cancer du côlon, comprenant

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(a) la détermination d'un niveau de TUCAN dans un échantillon contenant des cellules néoplasiques provenant d'un patient ayant un cancer avant traitement, et

(b) la détermination du niveau de TUCAN dans un échantillon contenant des cellules néoplasiques provenant dudit patient après traitement, de sorte que la comparaison dudit niveau de TUCAN avant traitement avec le niveau de TUCAN après traitement indique l'efficacité dudit traitement, dans lequel une diminution du niveau de TUCAN après traitement indique que le traitement est efficace.

16. Procédé selon la revendication 1, comprenant :

(c) la classification dudit patient comme appartenant soit à un premier groupe soit à un second groupe de patients, dans lequel ledit premier groupe de patients ayant des niveaux de TUCAN inférieurs au niveau de référence est classé comme ayant une probabilité accrue de survie en comparaison audit second groupe de patients ayant des niveaux de TUCAN supérieurs au niveau de référence.

17. Procédé selon les revendications 1 à 14 et 16, dans lequel ladite survie est une survie en rémission.

18. Procédé selon les revendications 1 à 14 et 16, dans lequel ladite survie est une survie sans maladie.

19. Procédé selon les revendications 1 à 18, dans lequel ledit échantillon est un tissu tumoral du côlon.

20. Procédé selon les revendications 1 à 18, dans lequel ledit échantillon est un fluide choisi dans le groupe constitué de sang, sérum, urine, sperme et selles.

21. Procédé selon les revendications 1 à 3, 15 et 16, dans lequel un niveau d'un polypeptide de TUCAN est mesuré.

22. Procédé selon les revendications 1 à 3, 15 et 16, dans lequel un niveau d'un polypeptide de TUCAN est mesuré en utilisant un anticorps spécifiquement réactif vis-à-vis d'un polypeptide de TUCAN.

23. Procédé selon les revendications 1 à 3, 15 et 16, dans lequel un niveau d'un acide nucléique de TUCAN est mesuré.

24. Procédé selon les revendications 1 à 23, dans lequel ledit patient a un cancer à un stade précoce.

25. Procédé selon la revendication 16, comprenant en outre:

(a) la détermination d'un niveau de cIAP2 dans ledit échantillon contenant des cellules néoplasiques provenant dudit patient ayant un cancer, et

(b) la classification dudit patient comme appartenant soit à un premier soit à un second groupe de patients, dans lequel ledit premier groupe de patients ayant des niveaux de TUCAN et des niveaux de cIAP2 inférieurs aux niveaux de référence est classé comme ayant une probabilité accrue de survie en comparaison audit second groupe de patients ayant des niveaux de TUCAN et des niveaux de cIAP2 supérieurs aux niveaux de référence.

26. Procédé selon la revendication 16, comprenant en outre :

(a) la détermination d'un niveau d'un biomarqueur choisi dans le groupe constitué de Apaf1, Smac et Bcl-2 dans ledit échantillon contenant des cellules néoplasiques provenant dudit patient ayant un cancer, et

(b) la classification dudit patient comme appartenant soit à un premier soit à un second groupe de patients, dans lequel ledit premier groupe de patients ayant des niveaux de TUCAN inférieurs au niveau de référence et des niveaux de l'un quelconque de Apaf1, Smac ou Bcl-2 supérieurs aux niveaux de référence est classé comme ayant une probabilité accrue de survie en comparaison audit second groupe de patients ayant des niveaux de TUCAN supérieurs au niveau de référence et des niveaux de l'un quelconque de Apaf1, Smac ou Bcl-2 inférieurs aux niveaux de référence.

27. Procédé selon la revendication 16, comprenant en outre la détermination d'un niveau d'un biomarqueur choisi dans le groupe constitué de cIAP2, Apaf1, Smac et Bcl-2 dans ledit échantillon contenant des cellules néoplasiques provenant dudit patient ayant un cancer avant et après traitement, dans lequel les niveaux du biomarqueur choisi et de TUCAN avant traitement sont comparés aux niveaux du biomarqueur choisi de TUCAN après traitement pour indiquer l'efficacité dudit traitement.

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**28.** Procédé selon les revendications 1 à 27, dans lequel le patient a un carcinome du côlon de stade II.

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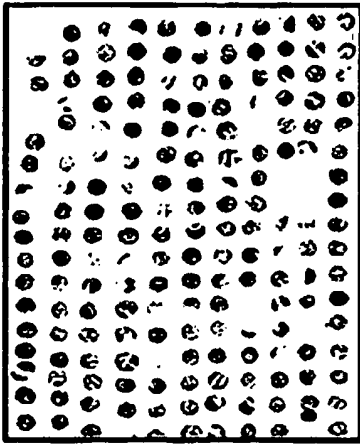
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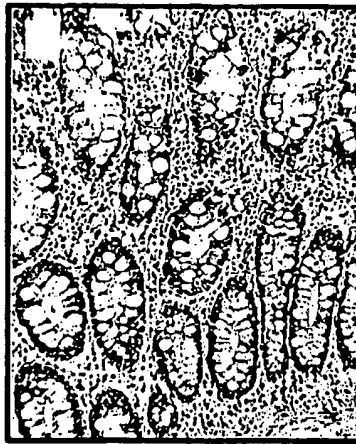
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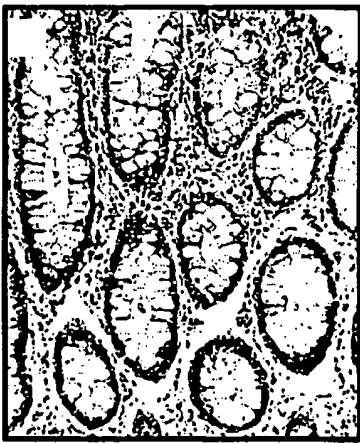
**FIG. 1A**



**FIG. 1B**



**FIG. 1C**



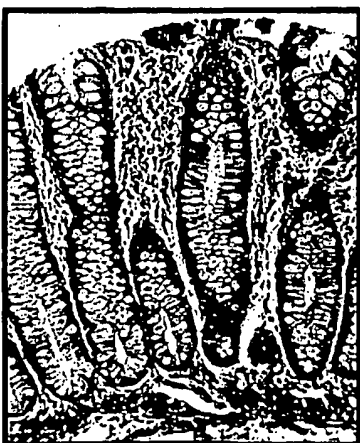
**FIG. 1D**



**FIG. 1E**



**FIG. 1F**



**FIG. 1G**



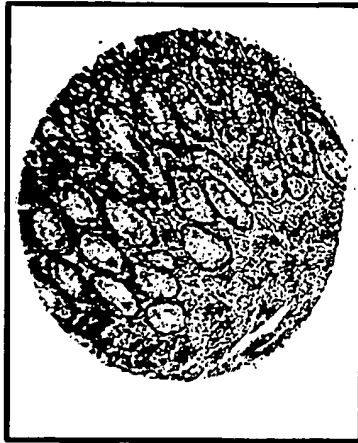
**FIG. 1H**



**FIG. 1I**



**FIG. 1J**



**FIG. 1K**



**FIG. 1L**



**FIG. 1M**



**FIG. 1N**



**FIG. 1O**



**FIG. 1P**

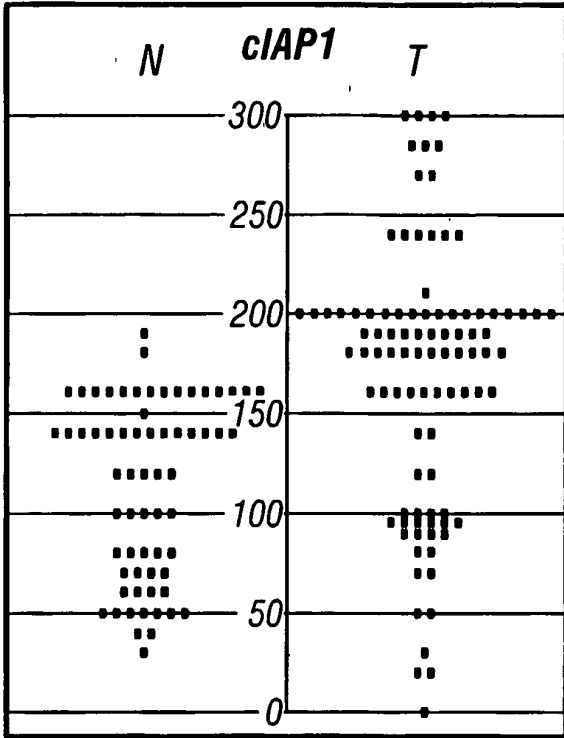


FIG. 2A

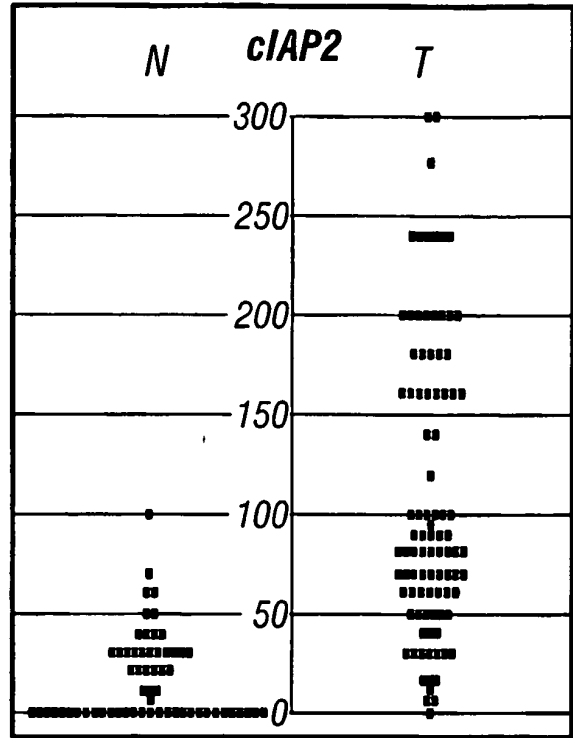


FIG. 2B

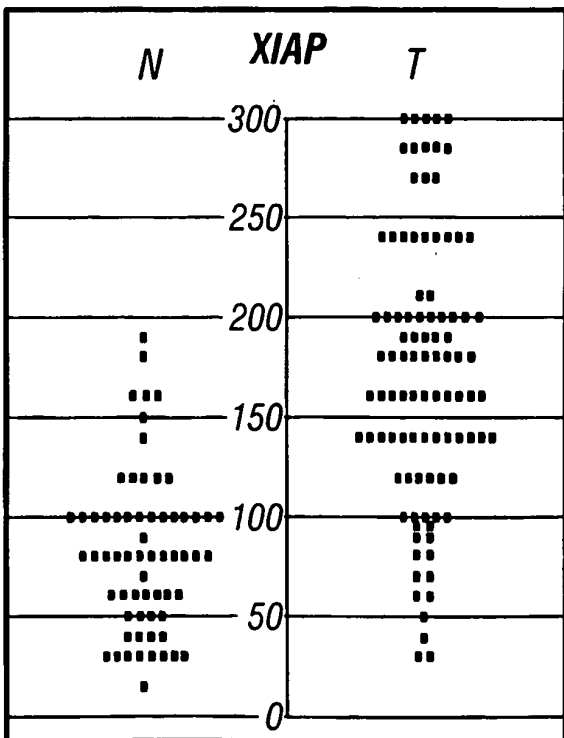


FIG. 2C

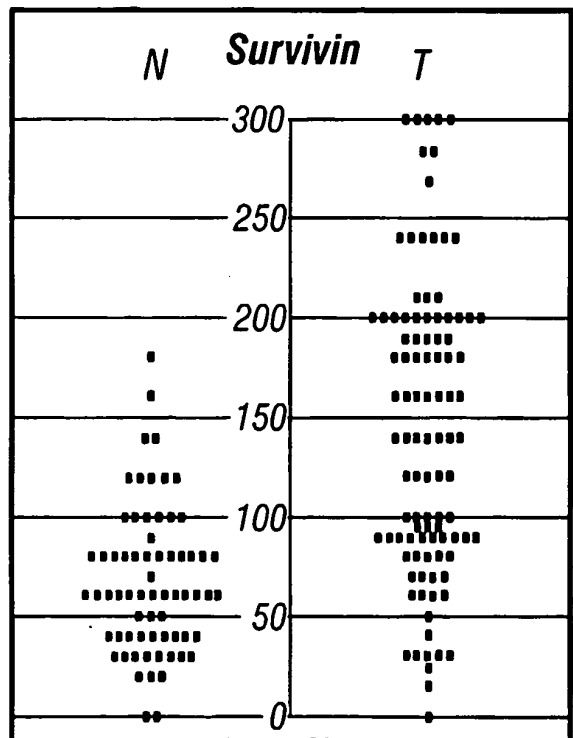
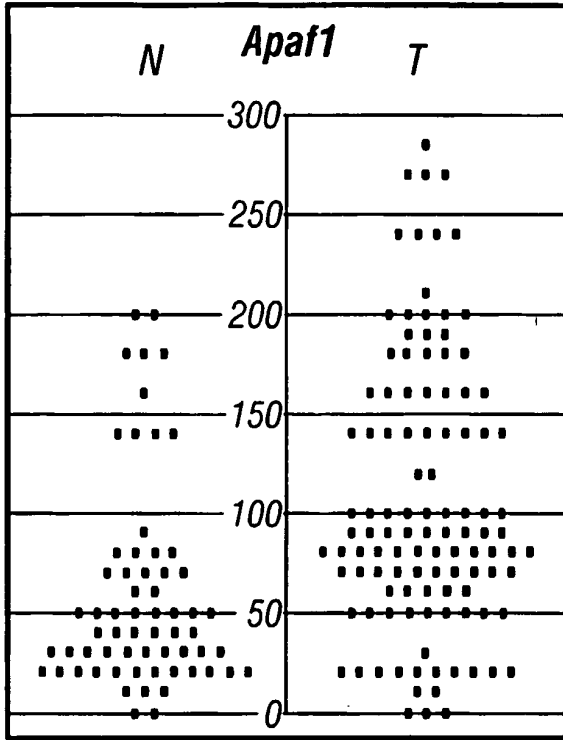
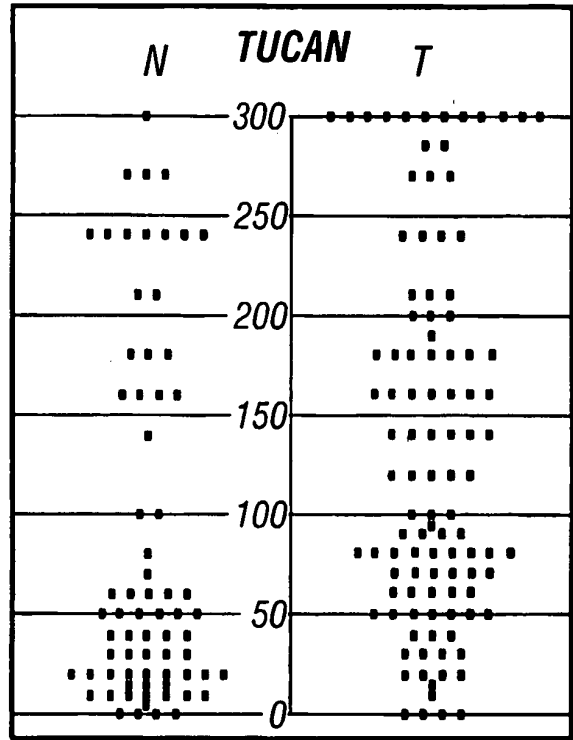


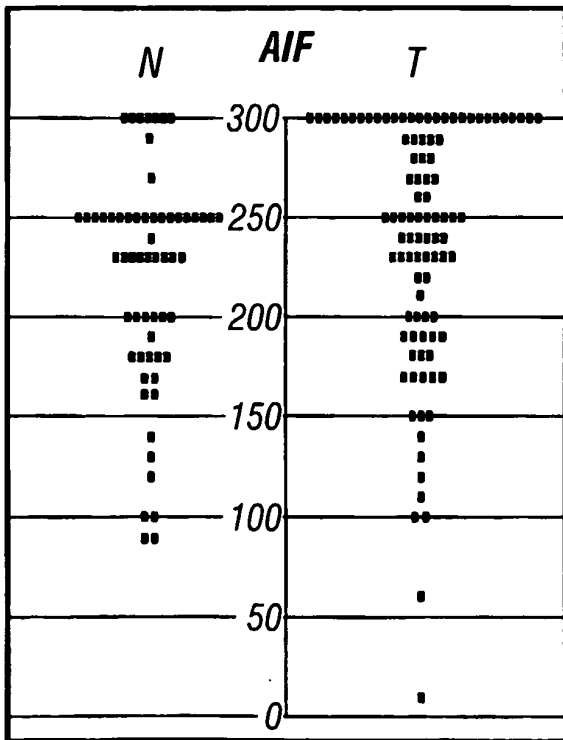
FIG. 2D



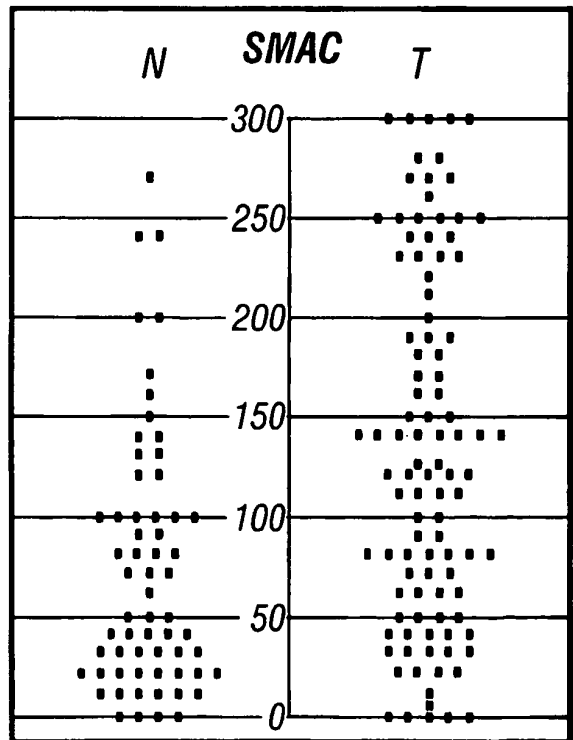
**FIG. 2E**



**FIG. 2F**



**FIG. 2G**



**FIG. 2H**

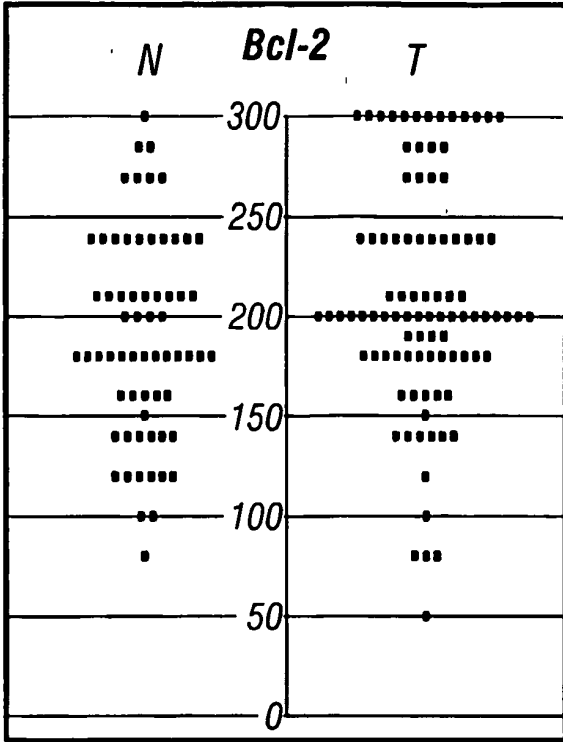


FIG. 2I

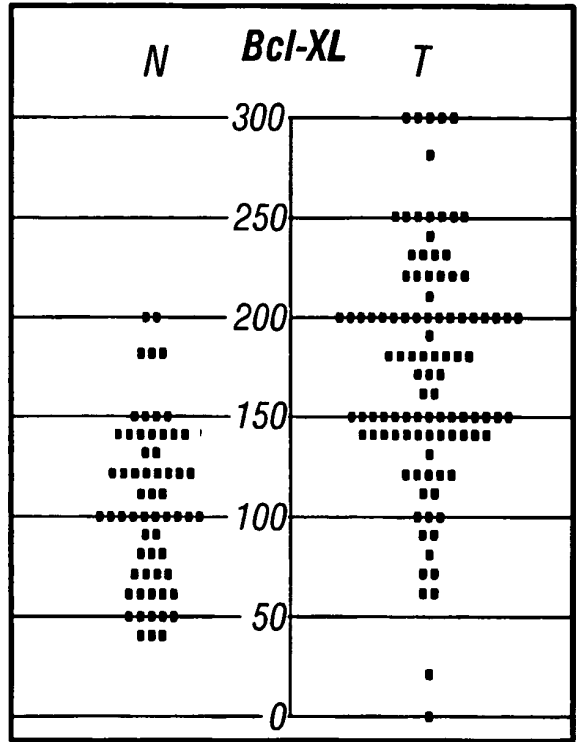


FIG. 2J

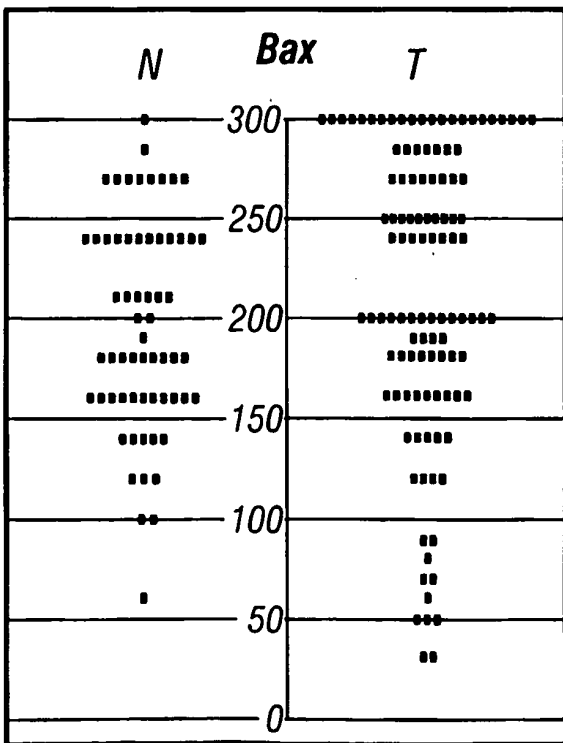


FIG. 2K

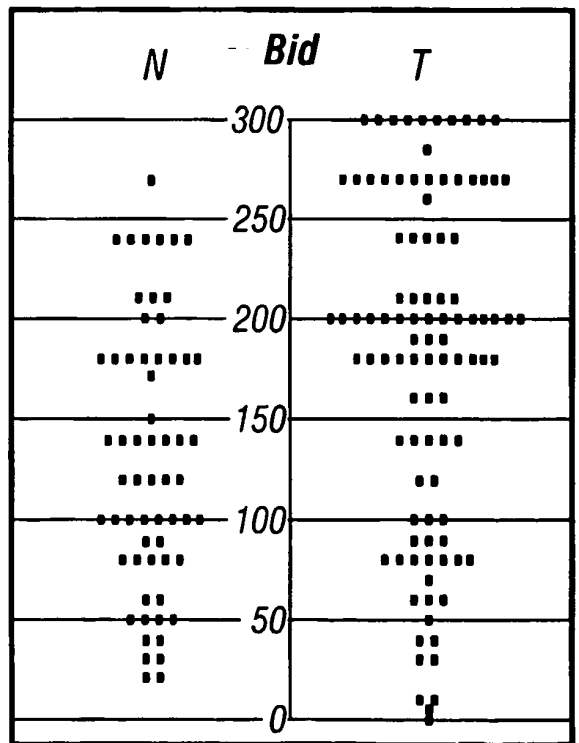


FIG. 2L

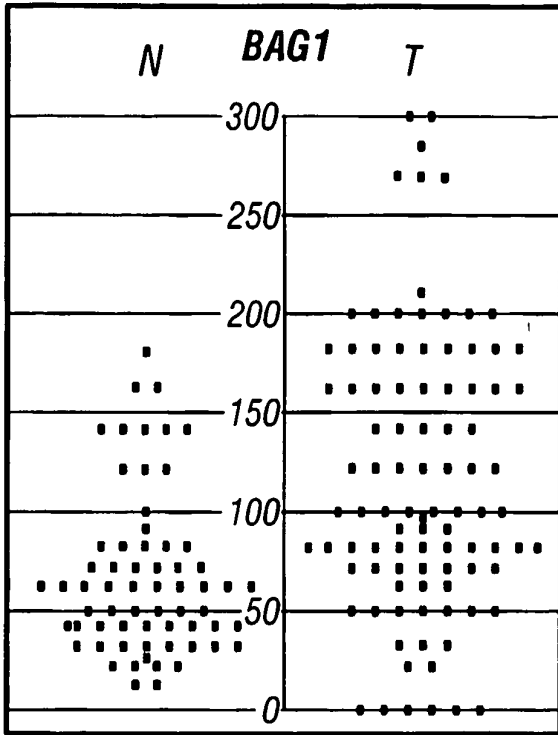


FIG. 2M

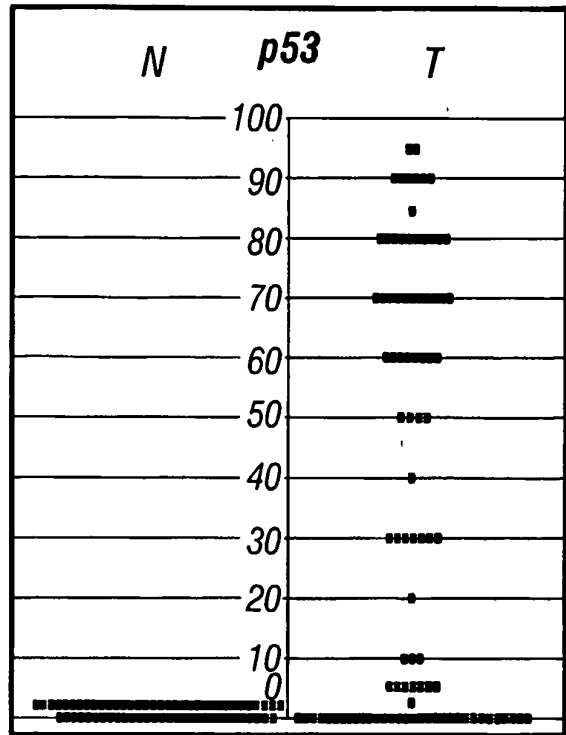


FIG. 2N

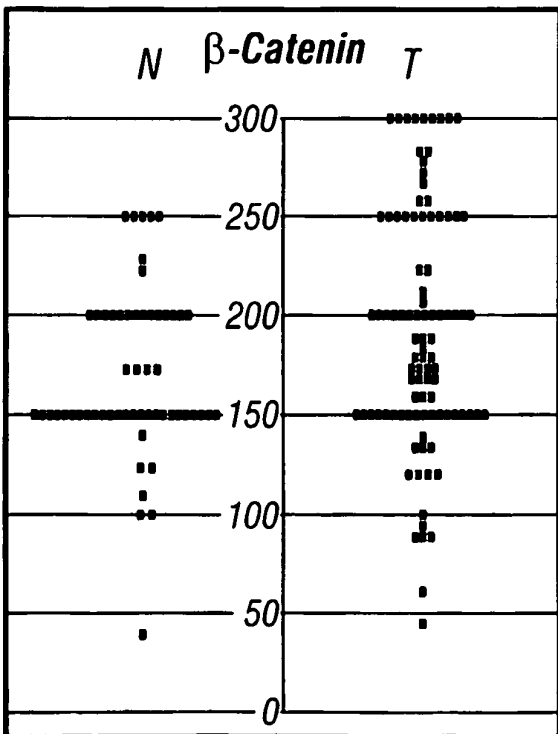


FIG. 2O

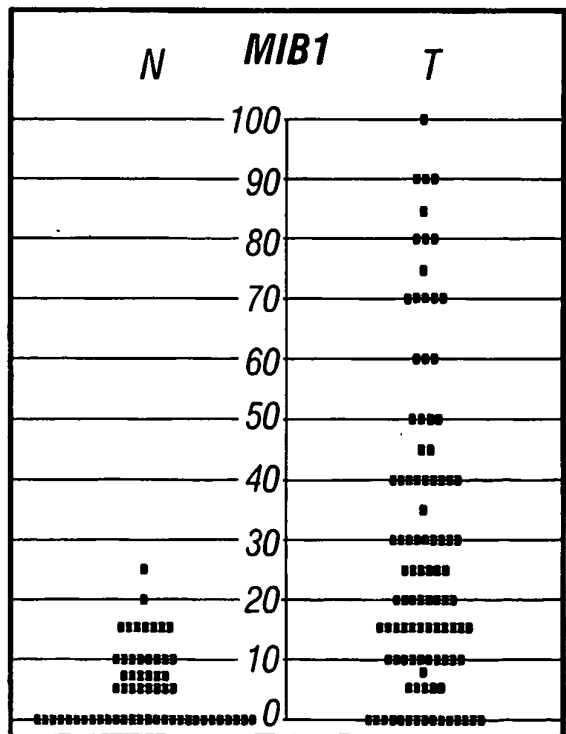


FIG. 2P

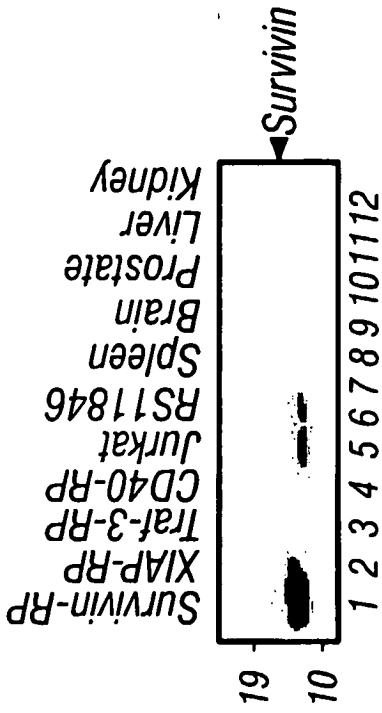


FIG. 3B

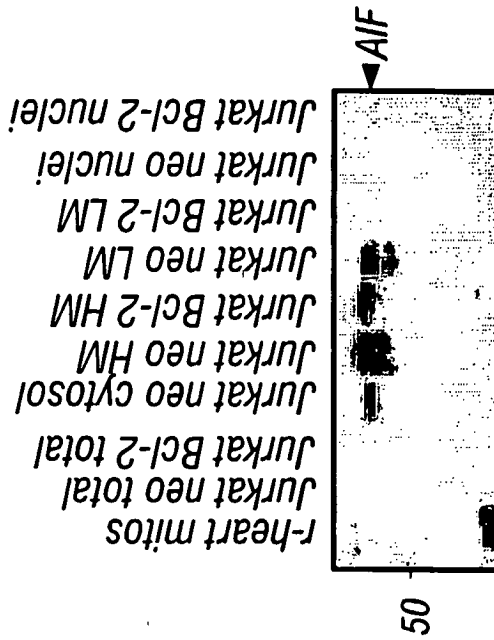


FIG. 3D

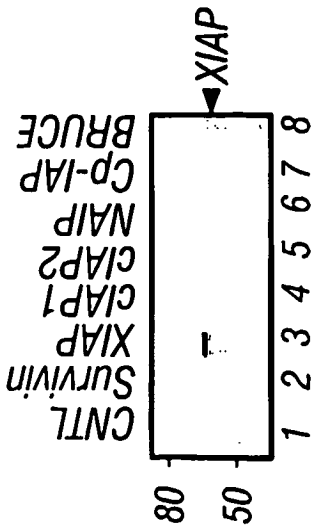


FIG. 3A

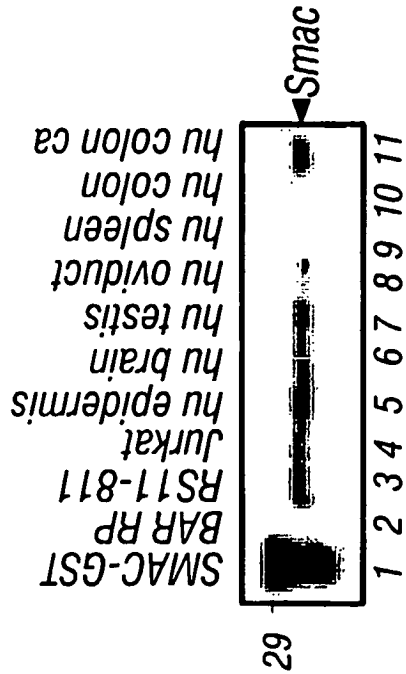


FIG. 3C

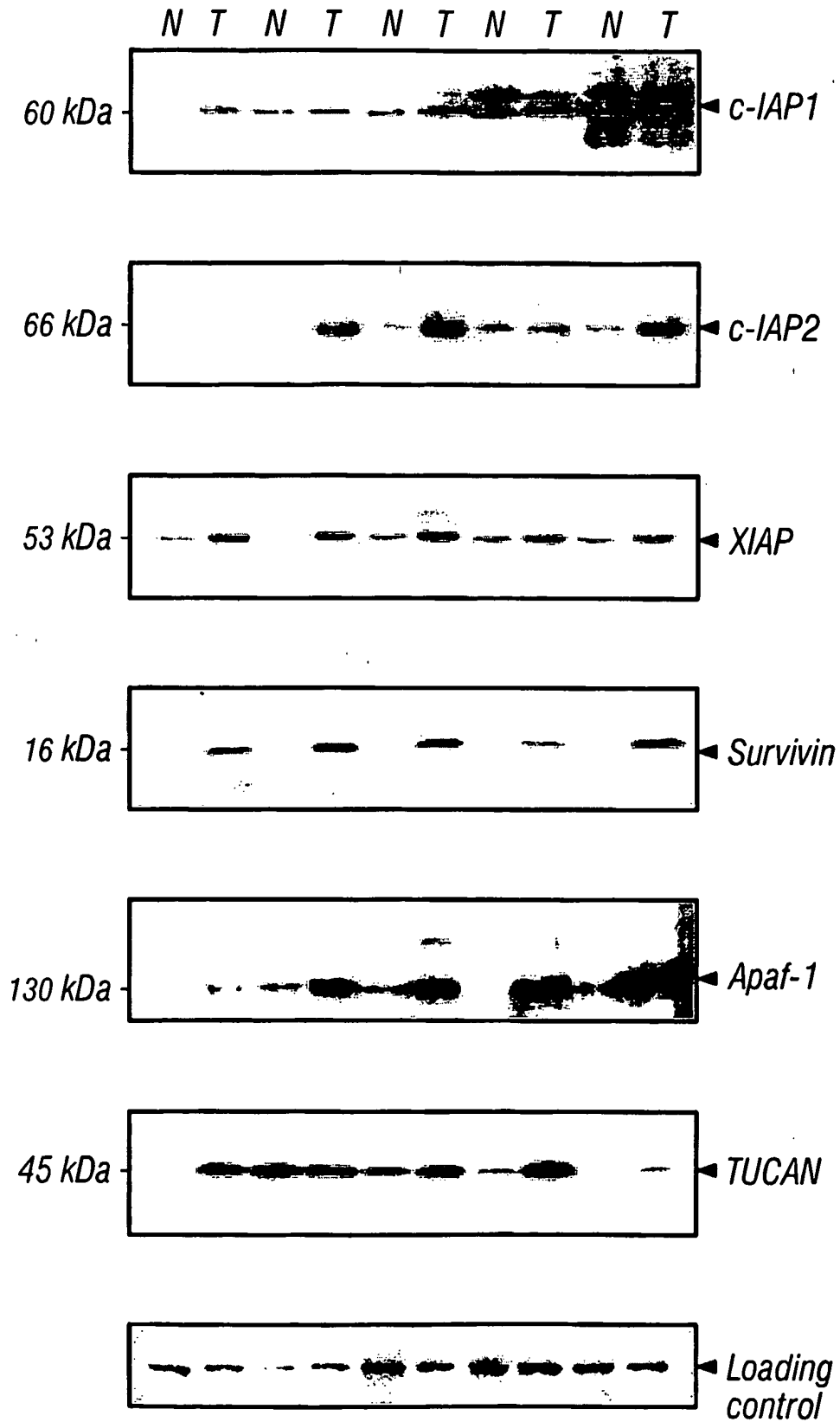


FIG. 3E

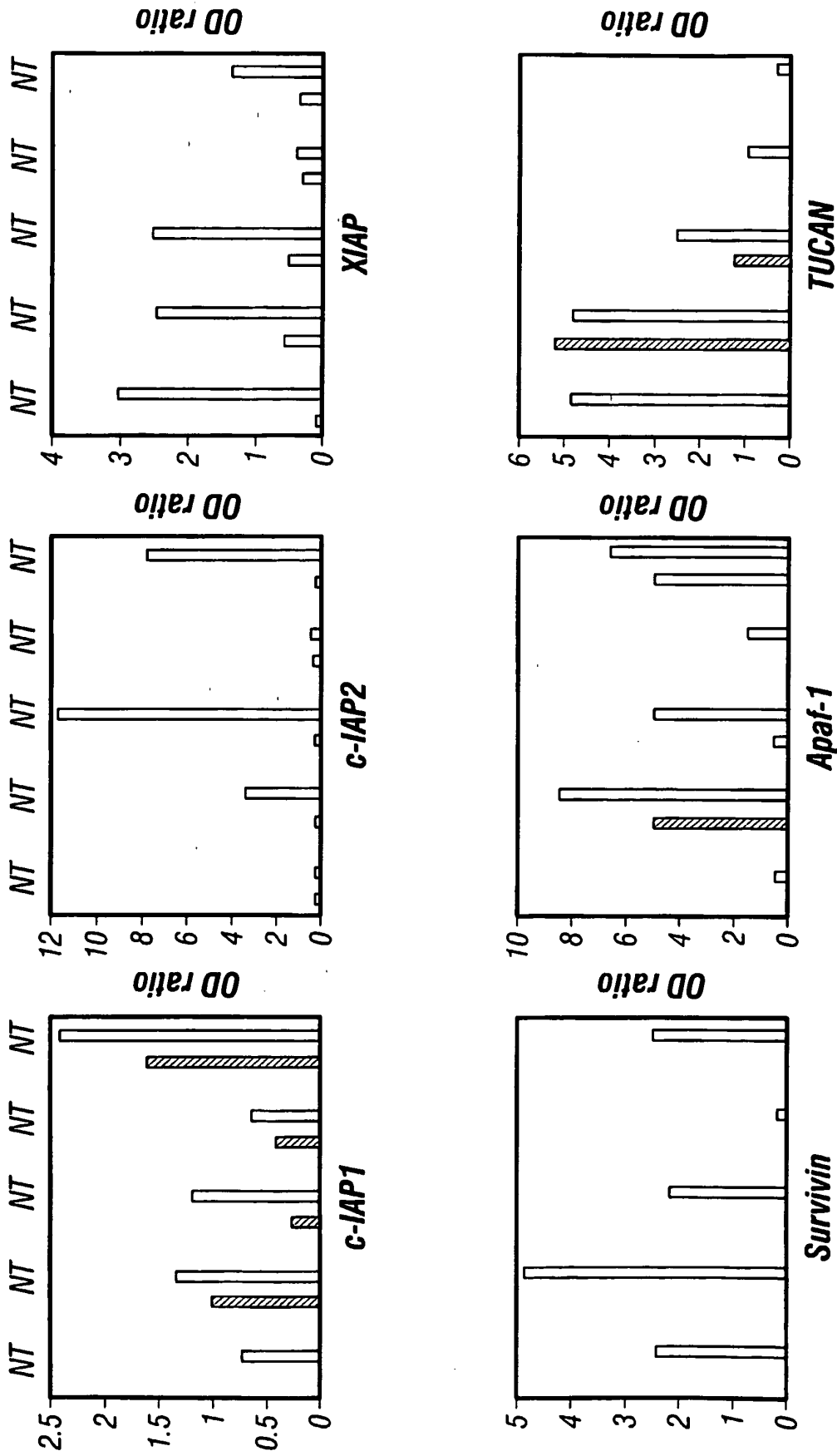
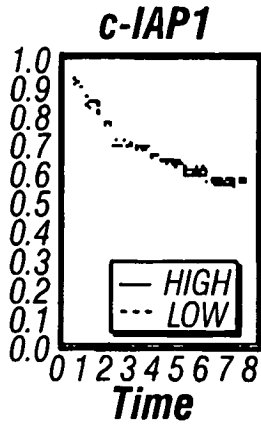
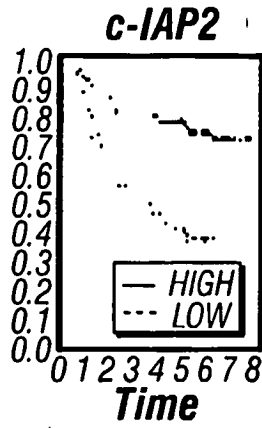


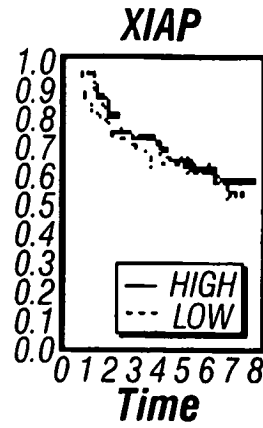
FIG. 3F



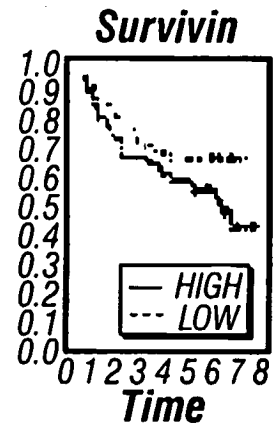
**FIG. 4A**



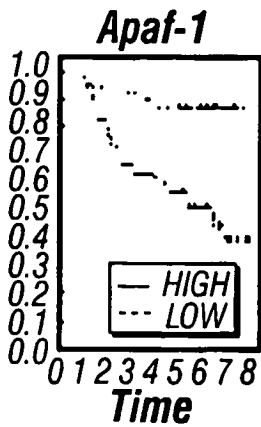
**FIG. 4B**



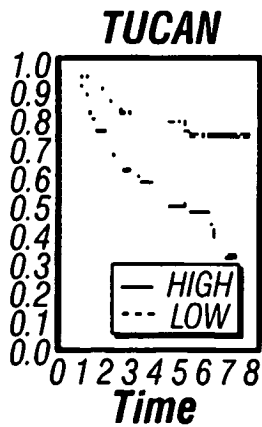
**FIG. 4C**



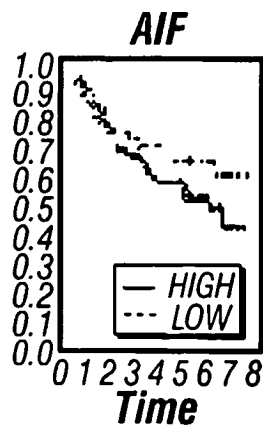
**FIG. 4D**



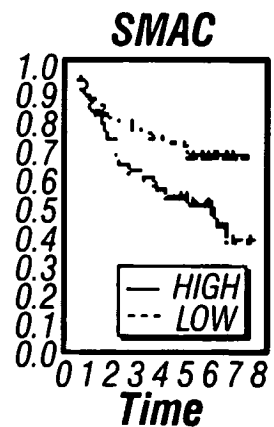
**FIG. 4E**



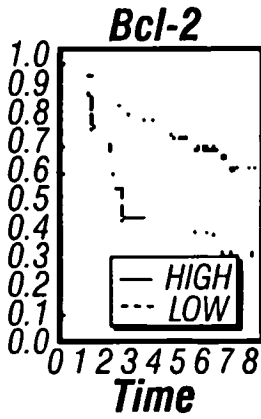
**FIG. 4F**



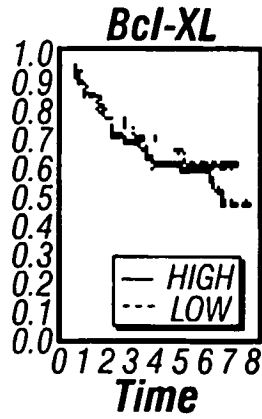
**FIG. 4G**



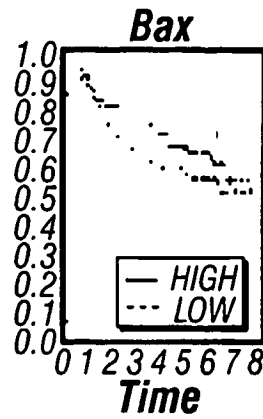
**FIG. 4H**



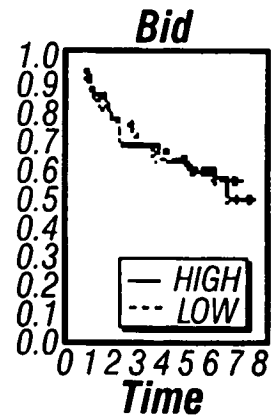
**FIG. 4I**



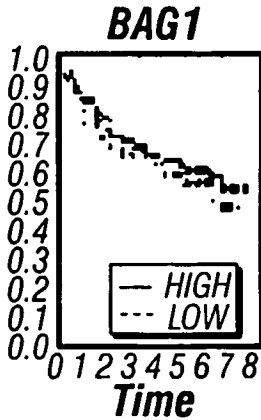
**FIG. 4J**



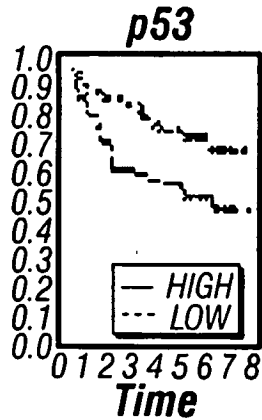
**FIG. 4K**



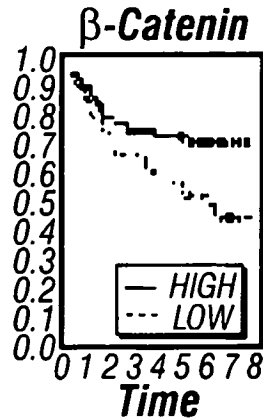
**FIG. 4L**



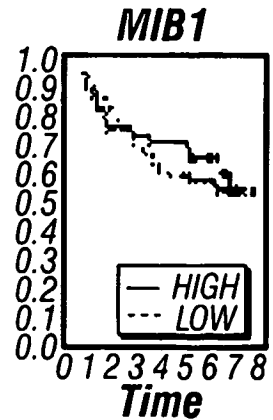
**FIG. 4M**



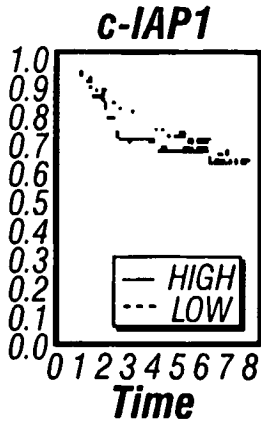
**FIG. 4N**



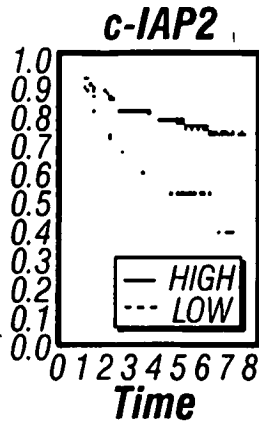
**FIG. 4O**



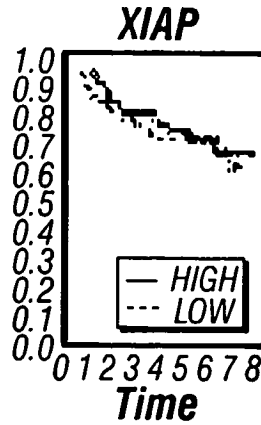
**FIG. 4P**



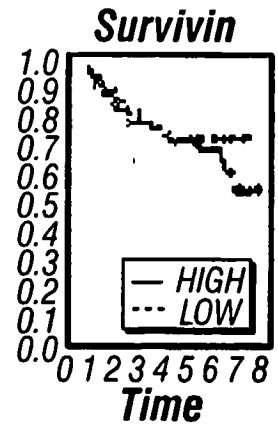
**FIG. 4Q**



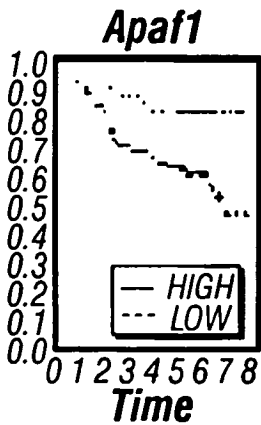
**FIG. 4R**



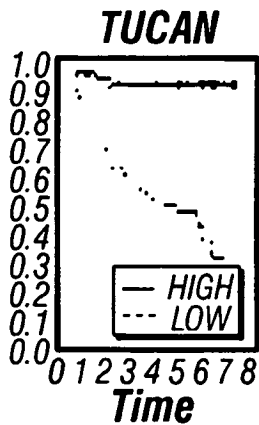
**FIG. 4S**



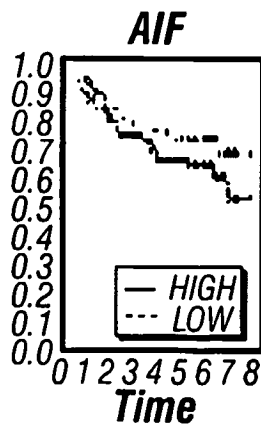
**FIG. 4T**



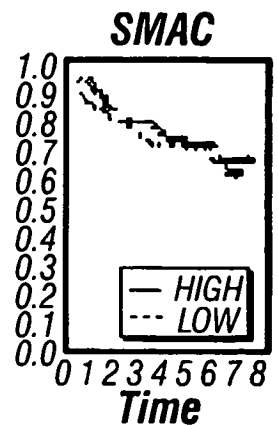
**FIG. 4U**



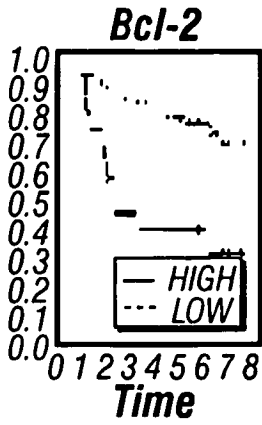
**FIG. 4V**



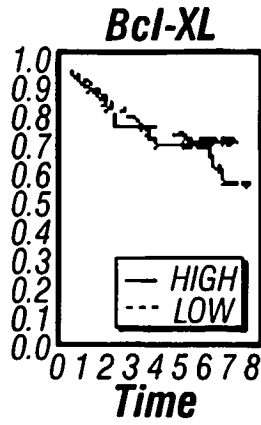
**FIG. 4W**



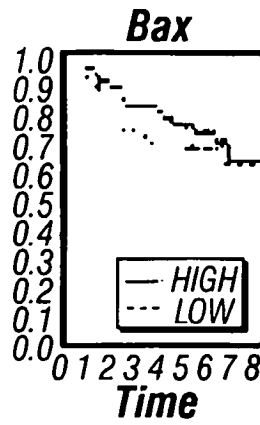
**FIG. 4X**



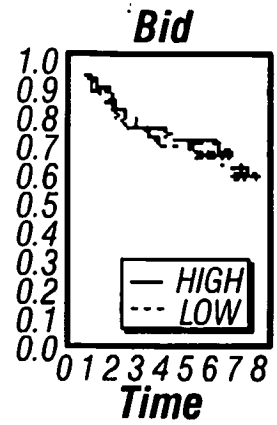
**FIG. 4Y**



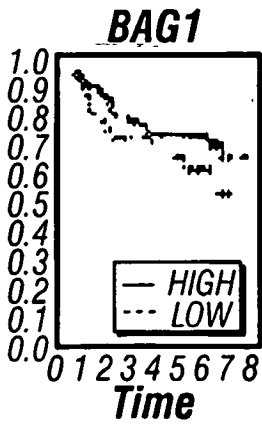
**FIG. 4Z**



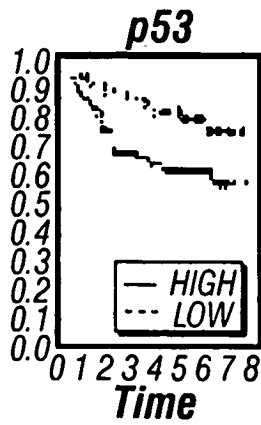
**FIG. 4A-1**



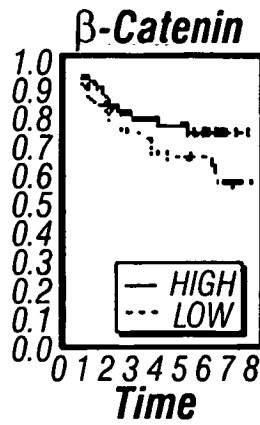
**FIG. 4B-1**



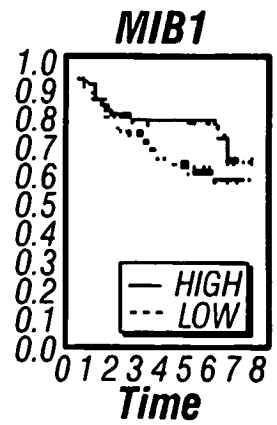
**FIG. 4C-1**



**FIG. 4D-1**



**FIG. 4E-1**



**FIG. 4F-1**

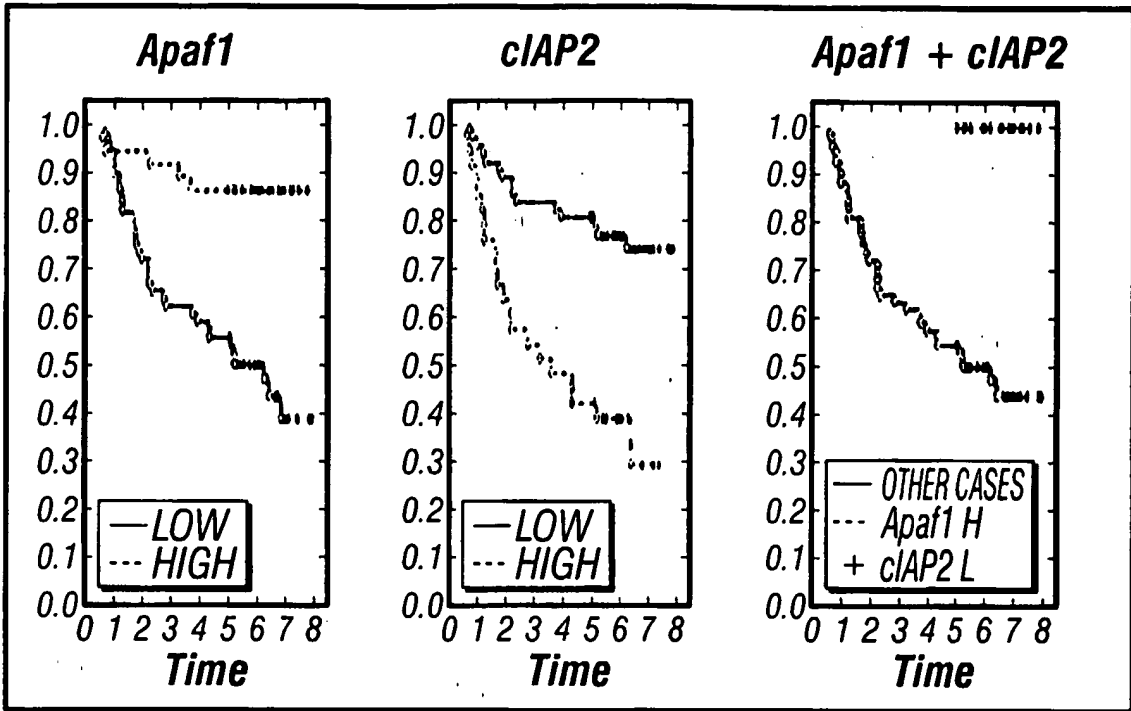


FIG. 5A

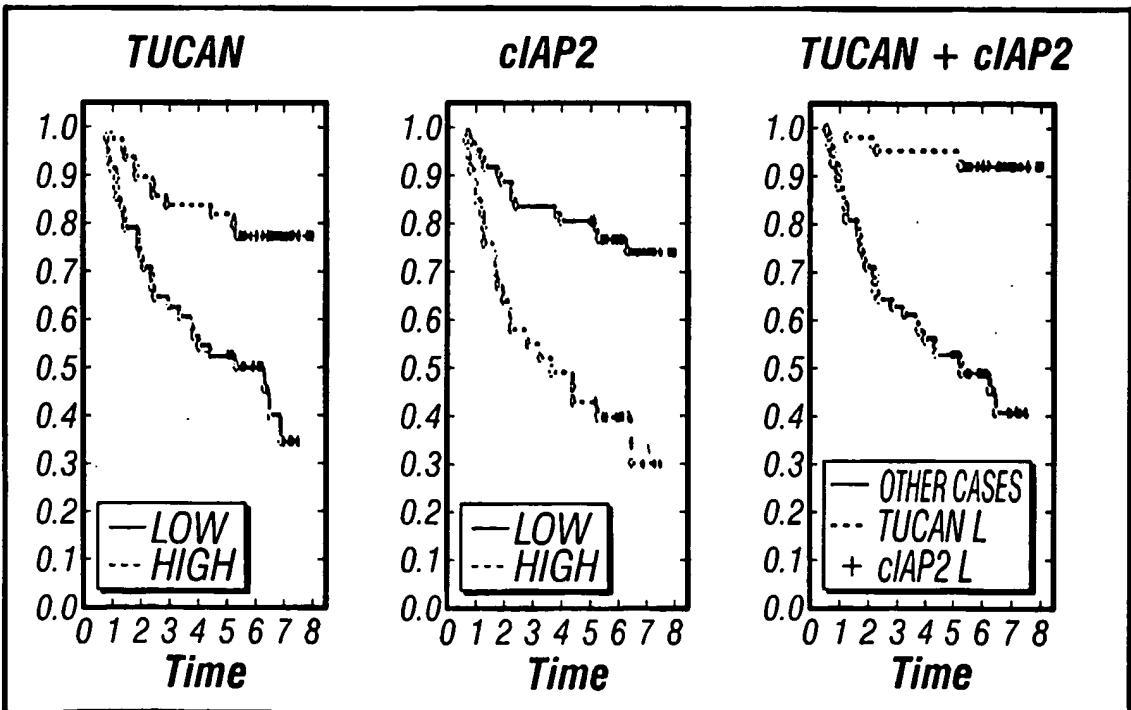


FIG. 5B

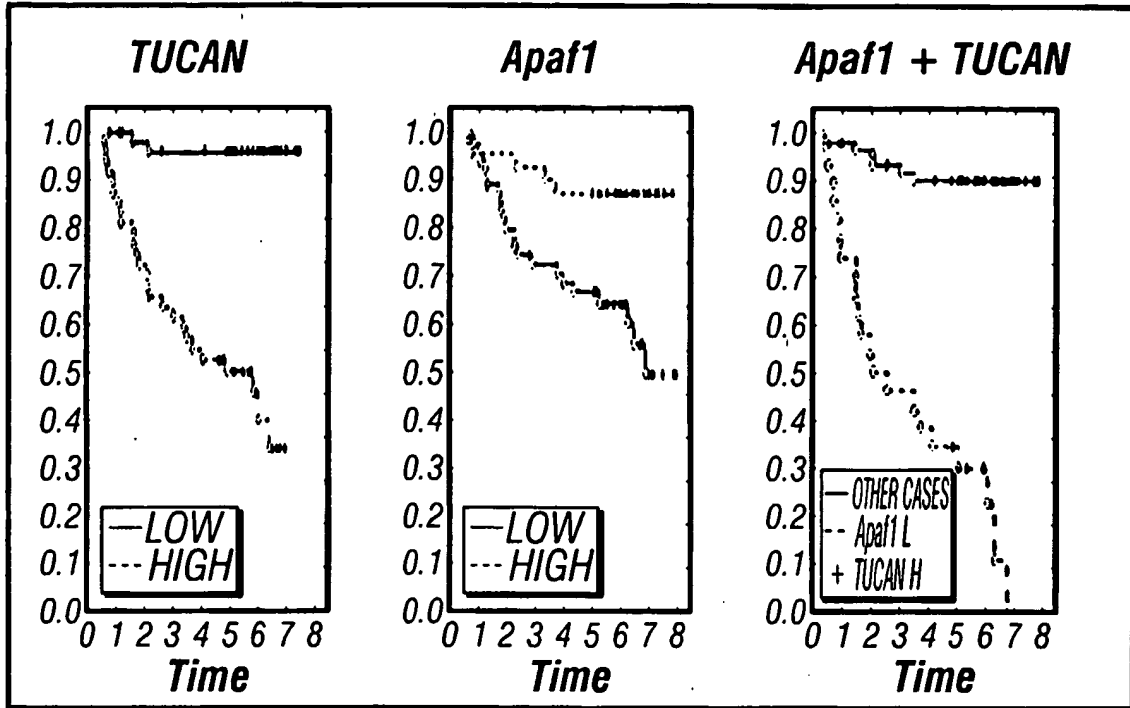


FIG. 5C

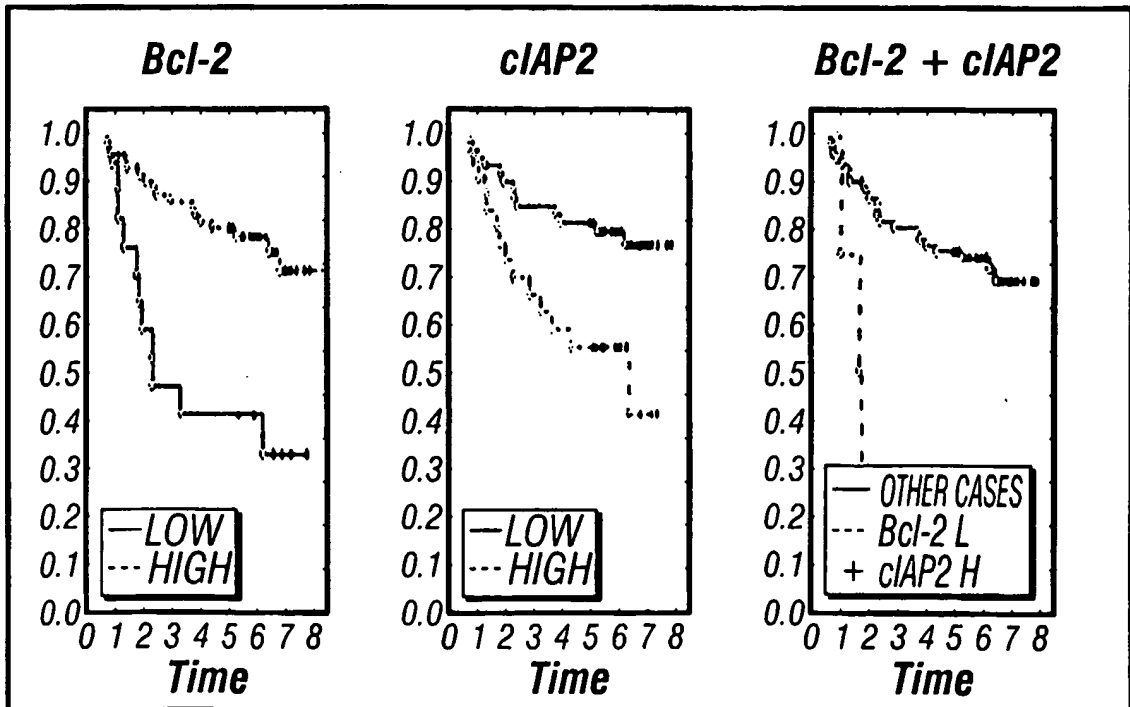


FIG. 5D

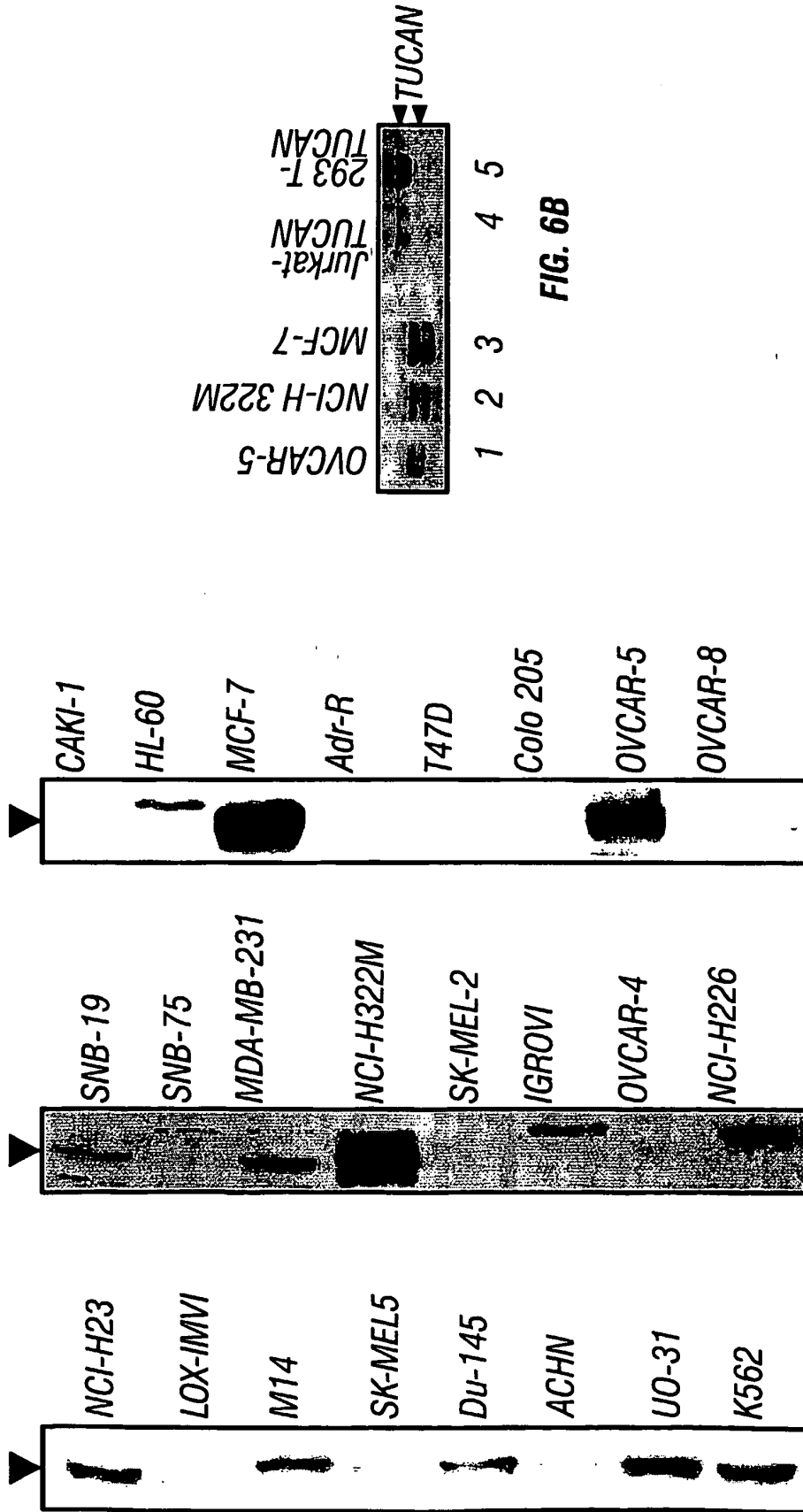


FIG. 6A

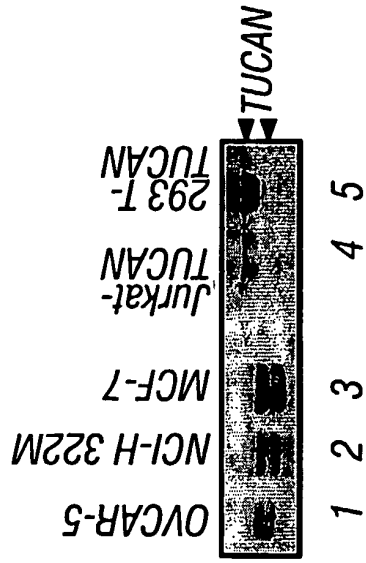
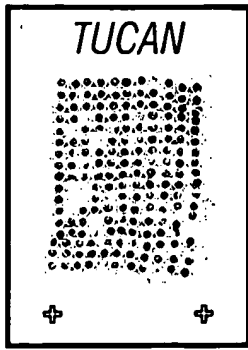
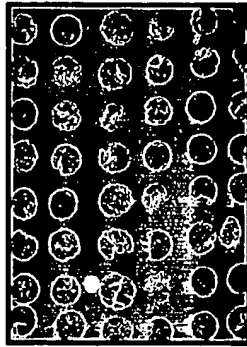


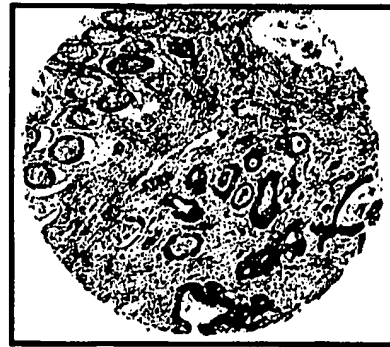
FIG. 6B



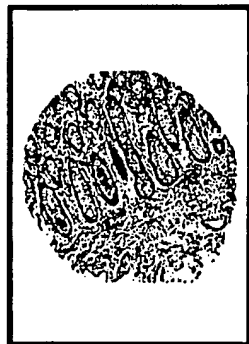
**FIG. 7A**



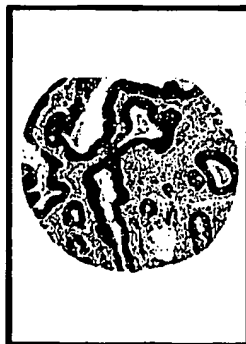
**FIG. 7B**



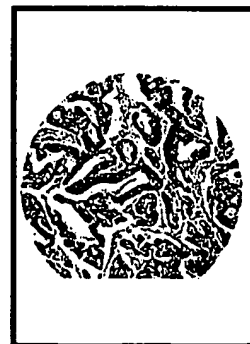
**FIG. 7C**



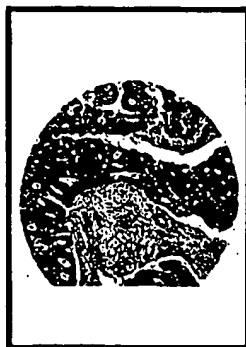
**FIG. 7D**



**FIG. 7E**



**FIG. 7F**



**FIG. 7G**

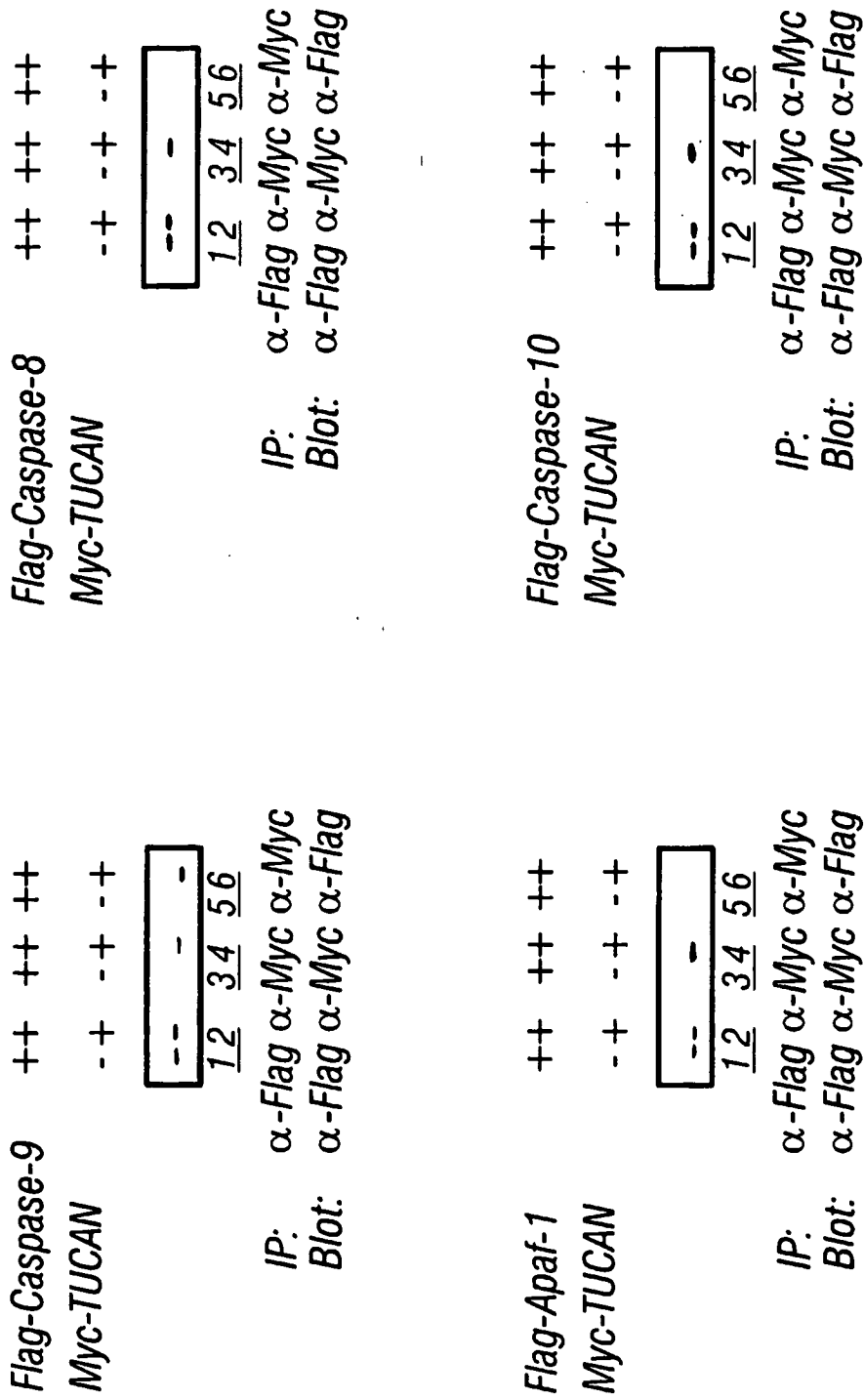
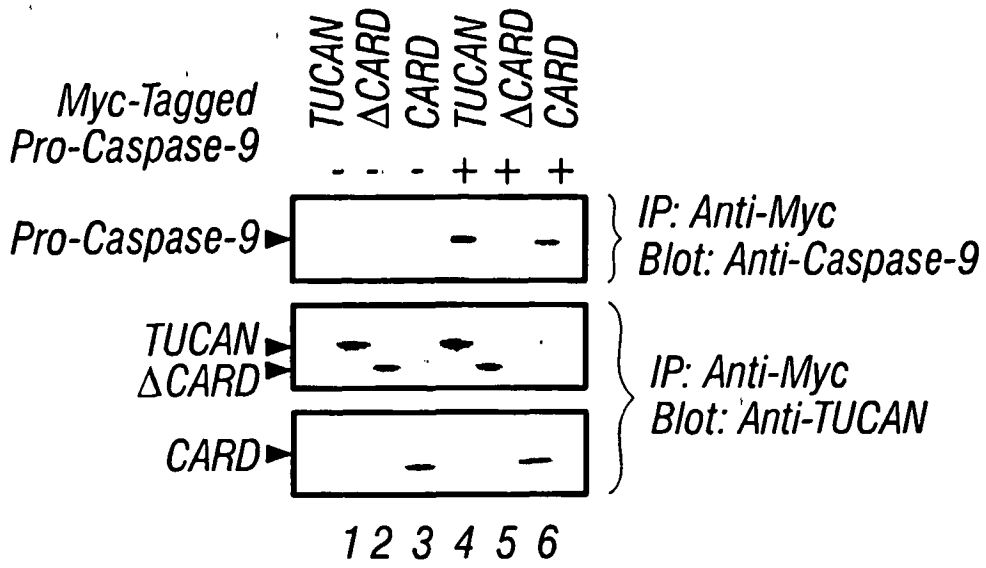
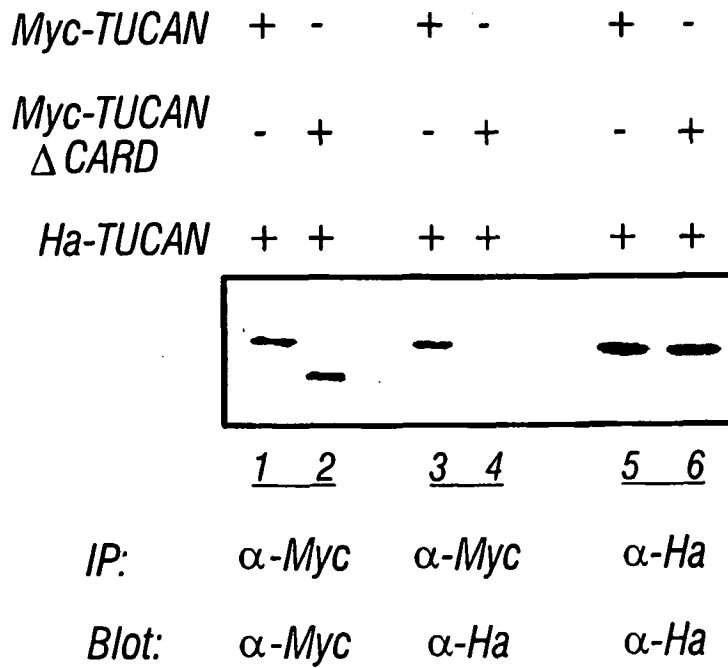


FIG. 8A



**FIG. 8B**



**FIG. 8C**

## REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	使用tucan确定癌症患者预后的方法		
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申请(专利权)人(译)	伯纳姆学院		
当前申请(专利权)人(译)	伯纳姆学院		
[标]发明人	REED JOHN C		
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IPC分类号	G01N33/574 G01N33/53 C12N15/09 C12Q1/68 G01N33/50		
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其他公开文献	EP1456645A4 EP1456645A2		
外部链接	<a href="#">Espacenet</a>		

摘要(译)

本发明提供了用于确定癌症患者的存活预后的方法。一种方法涉及 (a) 测量来自癌症患者的含有肿瘤细胞的样品中TUCAN的水平, 和 (b) 将样品中的TUCAN水平与TUCAN的参考水平进行比较, 其中TUCAN的水平低。该样本与患者的存活率增加相关。另一种方法涉及 (a) 测量来自癌症患者的含有肿瘤细胞的样品中的TUCAN水平, 和 (b) 将患者分类为属于第一组或第二组患者, 其中第一组患者具有低与具有高水平TUCAN的第二组患者相比, TUCAN水平被分类为具有增加的存活可能性。

Table 1: Types of Cancer

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