

## Detection Technologies

### A new broad-spectrum cancer marker

**A novel receptor protein shows promise for detecting a wide range of malignancies.**

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The statistics for cancer survival are sobering: One out of four people will ultimately die from some form of the disease, and of those who contract it, approximately 50% will not recover. In the United States, which represents just 5% of the world's population, this amounts to half a million deaths every year. The magnitude of this problem makes it easy to understand why so much money and effort is spent on developing new methods to detect cancer at earlier stages, when therapy results in a much higher percentage of cures.

Among the diagnostic tools developed during the past few decades, tumor markers provide a combination of low cost and accuracy that makes them suitable for diagnosis, monitoring, and screening. Despite a great deal of research in the area, only a handful of markers have found their way to clinical use. The most notable ones are alpha-fetoprotein (AFP), which appears in primary liver cancer and some rare forms of testicular cancer; carcinoembryonic antigen (CEA), which can help detect colorectal cancer; CA125, for ovarian cancer; and prostate-specific antigen (PSA), which appears in both prostate cancers and benign lesions.

This article presents data related to a new cancer marker, named RECAF, which exhibits high sensitivity and specificity on tissue sections and serum of patients with diverse types of malignancies.

#### Background

RECAF is a receptor protein that binds and takes up AFP, a circulating fetal protein. RECAF and AFP should not be confused; the latter is a liver and testicular cancer marker known since 1963 whereas RECAF is an emerging broad-spectrum cancer marker.<sup>1</sup>

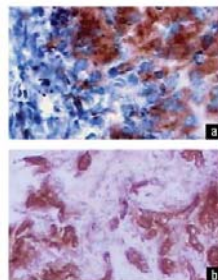


Figure 2. Frozen sections of two different breast ductal carcinomas stained with

AFP works in the fetus as albumin does in the adult: Both bind and carry other, smaller molecules, including fatty acids.<sup>2</sup> In vitro experiments have shown that after binding to RECAF, AFP penetrates the cell via coated pits, releases its load of fatty acids, and then leaves immunologically intact, likely ready to fetch another fatty acid molecule.<sup>3-5</sup> The uptake of AFP, and hence the expression of RECAF, is related to the degree of cell differentiation: When a given fetal organ or tissue has reached a certain maturity, AFP is no longer taken up even if it is present in high concentrations in the extracellular fluid.<sup>6-8</sup> Since cancer cells are poorly differentiated, it was first postulated and then demonstrated that they reexpress RECAF; hence, RECAF behaves as an oncofetal antigen.<sup>9,10</sup> The AFP uptake by fetal cells occurs in most organs and tissues; therefore,

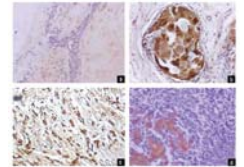


Figure 1. Histo-RECAF on breast tumors. Fibroadenoma (negative), 100x (a); Ductal carcinoma, 200x (b); Lobular carcinoma, 100x (c); Axillary lymph node metastasized by a breast carcinoma, 100x (d) (click to enlarge).

[Cryo-RECAF. Counterstained with Haematoxylin 100¥, \(a\); Without Haematoxylin, 200¥ \(b\) \(click to enlarge\).](#)

RECAF, which mediates the uptake, has been shown to be reexpressed in many types of cancers.<sup>11-16</sup>

RECAF is not a single molecule: There are two main membrane fractions, with molecular weights of 18 and 31 kDa, and two main soluble cytoplasmic components, weighing ~50 and ~62/67 kDa, respectively. The latter often appears as a doublet.<sup>17</sup> Each of these fractions binds AFP. The AFP binding site is localized in the sugar moiety of these glycoproteins. This has been demonstrated by experiments in which the AFP binding activity is fully conserved following complete digestion of RECAF with pepsin; after treatment with sodium periodate (in preparation), on the other hand, it is completely destroyed.

The presence of a membrane RECAF fraction can be used for several purposes, from specifically targeting cancer cells to tumor imaging. In reference to the latter, researchers at Pacific BioSciences Research Centre (Richmond, BC) have imaged breast tumors as small as 4 mm in mice and 1.5 cm in humans using radiolabeled AFP, which is a homologous protein and, unlike antibodies, does not need to be humanized to prevent an immune response from the host.<sup>18</sup>

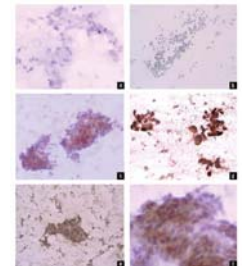


Figure 3. Breast fine-needle biopsies stained for RECAF. Fibroadenomas, 200¥ (a, b); Carcinomas, 200¥ (c–e); A carcinoma, 400¥ (f) (click to enlarge).

The existence of soluble RECAF fractions has created the opportunity to detect them in the cytoplasm of tissue sections as well as in the serum of cancer patients.

### RECAF in Tissue Sections

To detect RECAF, staining kits were developed that use peroxidase-labeled human AFP to bind to the protein. FDA has classified this assay, named Histo-RECAF, as a Class I staining device for paraffin sections of breast tissue. A variant of the test called Cryo-RECAF has been engineered to stain frozen sections in approximately 15 minutes, while the patient is under anesthesia. FDA has not yet cleared Cryo-RECAF, and therefore the test is available for research purposes only.

The kits contain all reagents and solutions required for staining. Histo-RECAF contains enough material for 60 slides, whereas the Cryo-RECAF kit contains 10 slides and is designed for use on a single patient. Since the RECAF binding ligand is labeled, the staining is conducted in only one step followed by development with diaminobenzidine, which is included in the package as a ready-to-use solution. Frozen sections require a three-minute incubation in Carnoy's fixative (25% acetic acid in 95% ethanol) prior to staining.

The large majority of cancers stain positively (e.g., breast cancers are all positive). Normal tissue and benign tumors are practically all negative, with the odd RECAF-positive cell. Figure 1 shows the staining, in brown, of breast tissues with Histo-RECAF. Both ductal and lobular carcinomas are positive, whereas fibroadenomas and other benign lesions are negative. Figure 1d shows the staining of breast cancer cells in an infiltrated sentinel axillary lymph node. The contrast offered by the staining may prove useful in cases where the number of cancer cells is too low to be easily visualized.

Figure 2 shows frozen sections of two breast cancers stained with Cryo-RECAF. Here, the staining should prove useful in distinguishing the cancer cells in cases where the uncertainty of the diagnosis is compounded by the inevitable poor quality of frozen sections. Fine-needle biopsies of the breast are another situation in which diagnosis can prove difficult due to a lack of tissue structure.

Figure 3 shows the staining difference between benign and malignant cases using

Cryo-RECAF with a higher dilution of AFP-peroxidase to reduce the background, and a longer incubation time.

Cancer Type	Sensitivity with 95% specificity (%)	Sensitivity with 99% specificity (%)	Number
Ovarian	88	82	152
Breast	88	87	27
Lung	84	87	22
Bladder	83	80	48
Prostate vs. normal	88	88	28
Total	84	83	233
Blind Range*	0	0	22
Prostate benign†	88	8	22

Table I. All samples were compared with a set of 103 samples from individuals without tumors (normal individuals). The cutoff value was extracted from the normal individuals to include either 95 or 99% of cases. The sensitivity values in the columns above correspond to the percentage of known cancers with values over the corresponding cutoff values (click to enlarge).

Figure 4 shows examples of other RECAF-positive malignancies. The results obtained with anti-RECAF antibodies are similar, except that the staining is cleaner and the background is weaker.

Interesting enough, there seems to be a correlation between the degree of cell malignancy and staining: The more aggressive a tumor appears under the microscope, the more intense the RECAF staining. This is also apparent within a given sample. Loose, invading cords of cancer cells are usually more positive than cancer cells in organized structures such as neoducts, glands, etc.

### RECAF in Serum

The soluble fractions of RECAF can be released from cancer cells either actively or after the cells die. Therefore, the circulating RECAF in cancer patients may be higher than in noncancerous individuals. To test this hypothesis, researchers at Pacific BioSciences Research Centre developed a competitive radioimmunoassay using a polyclonal rabbit antibody reactive against both the 50-kDa and the 62/67-kDa soluble RECAF fractions. The purified IgG fraction of the antiserum is coated onto 96-well plates, which are then blocked with

3% bovine serum albumin. Soluble RECAF, purified from cancer cells in culture and labeled with 125I, is mixed with the patient's sera (50 µl), and the mixture is incubated in the wells coated with the anti-RECAF antibody. After one hour, the wells are washed and detached from the plate's frame, and the radioactivity is measured in a gamma counter. A calibrated cell extract containing RECAF is used as a standard and the determination is expressed in RECAF units.

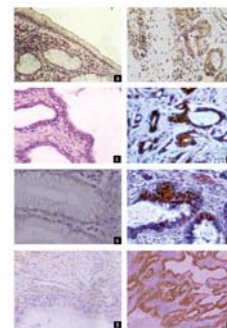


Figure 4. Different tissues stained with Histo-RECAF. Normal stomach, 40% (a); Stomach cancer, 100% (b); Prostate adenoma, 40% (c); Prostate adenocarcinoma, 100% (d); Normal colon, 200% (e); Colon carcinoma, 100% (f); Normal lung, 40% (g); Lung cancer, 40% (h). (click to enlarge).

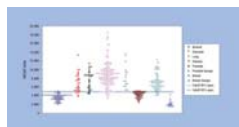
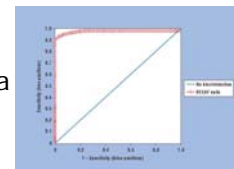


Figure 5. Distribution of RECAF values for normal, cancerous, and benign tumor samples. The horizontal lines mark the 95 and 99% specificity cutoff values (click to enlarge).

The results presented in this paper were obtained with the 62/67-kDa fraction and are similar to the results obtained using the purified 50-kDa fraction. Figure 5 shows the distribution of values for normal individuals, patients with benign lesions, and cancer patients. The horizontal lines represent the cutoff values for 95 and 99% specificity (i.e., only 5 or 1% of the normal samples fall above the cutoff value). Table I shows the corresponding sensitivity value and the number of samples of each type of malignancy or benign tumor. It is worth noting that a small number of prostate tumors appear as false positives when using the 95% specificity cutoff value. Increasing the cutoff value to yield 99% specificity greatly reduces the number of false positives at the expense of reducing, by a small percentage, the overall

sensitivity of the test. Figure 6 shows the receiver operating characteristic (ROC) analysis of all the cancer patients combined, against a combination of all the benign and normal cases. This graph gives an idea of what to expect when the serum-RECAF test is used for screening. The individual ROC curves for each type of cancer are similar in shape and area (data not shown).

For ovarian cancers, a relatively high correlation between circulating CA125 and RECAF ( $r^2 = 0.81$ ) was found. On the other hand, there was no correlation with CEA in a small number of colon cancers (data not shown). The origin of that correlation is uncertain since CA125, which has an MW higher than 200 kDa, appears to be unrelated to RECAF (MW < 70 kDa).



## Conclusion

Most cancer markers have been discovered by making antibodies that react against cancer tissue but not against normal or benign tissue. RECAF is an exception in that it was first described as part of a physiological mechanism during embryogenesis, and then proposed as a new cancer marker. The final assessment of any assay comes from evaluating its performance on a large number of samples, but it is worth noting that the available basic research data support the concept that RECAF behaves as a broad-spectrum oncofetal antigen.

Histo-RECAF is designed as a tool to pinpoint cancer cells under the microscope, particularly when searching for lymph node metastases or in frozen sections and fine-needle biopsies. A clear difference emerges when staining cancer cells and normal or benign cells. As a result, Histo-RECAF might also prove useful in testing for cancers such as thyroid carcinomas, which are difficult to diagnose by needle biopsy.

When used with automated systems employing vision recognition, the test can also drastically reduce computer time by examining only the brown spots (cells) on the slide. This could be beneficial for applications such as cervical smears (PAP smears), which are carried out approximately 50 million times each year. Paraffin sections of cervical cancers are positive, in contrast to the normal cervical epithelium, which is negative. This indicates that the test should work. However, before this test can be used systematically for cervical cancer diagnosis, the intense background emerging from the poor conditions of the cells when collected needs to be addressed. The same applies to sputum samples tested for lung cancer.

In serum for all types of cancer studied (breast, prostate, lung, stomach, and ovary), RECAF values were elevated. Together, these represent about 50% of all cancers in the Western hemisphere. The assay can detect approximately 90% of lung and breast cancers, the two prevalent types of malignancies and those for which most current markers perform poorly.

Since a consistently negative type of cancer has not yet been found, it is safe to assume that the assay should work for other cancers as well. For instance, the vast majority of benign tumors studied were RECAF negative, which is advantageous for prostate cancer diagnosis since PSA tends to be elevated in benign prostate tumors. Thus, the combination of a cancer-specific marker, such as RECAF, with a tissue-specific marker, such as PSA, could improve the specificity of the latter.

The behavior of RECAF as a sensitive and specific pan-cancer marker makes it a suitable candidate for routine screening; and because only one test is required, the cost is minimal. Routine screening leads to earlier detection, and this is, of course, closely linked to survival.

The results of this investigation indicate that RECAF has the potential to become a cancer marker of clinical significance. To assess the full extent of this potential, more samples from these and other types of cancer, as well as from benign lesions, must be studied.

## References

01. GI Abelev et al., "Production of Embryonal Alpha-Globulin by Transplantable Mouse Hepatomas," *Transplantation* 1 (1963): 174–180.
02. M Calvo et al., "Fatty Acids Bound to Alpha-Fetoprotein and Albumin during Rat Development," *Biochimica et Biophysica ACTA* 15 (1988): 238–246.
03. HC Lorenzo et al., "Alpha-Fetoprotein Binding and Uptake by Primary Cultures of

Figure 6. ROC analysis of 103 normal individuals and 333 assorted cancer samples. The area under the curve is 0.988. Overall sensitivity is 94% with 95% specificity, or 91% with 99% specificity (click to enlarge).

Human Skeletal Muscle," *Tumour Biology* 17 (1996): 251–260.

04. M Geuskens, J Naval, and J Uriel, "Ultrastructural Studies of the Intracellular Translocation of Endocytosed Alpha-Fetoprotein (AFP) by Cytochemistry and of the Uptake of 3H-Arachidonic Acid Bound to AFP by Autoradiography in Rat Rhabdomyosarcoma Cells," *Journal of Cellular Physiology* 128 (1986): 389–396.

05. J Uriel, J Naval, and J Laborda, "Alpha-Fetoprotein-Mediated Transfer of Arachidonic Acid into Cultured Cloned Cells Derived from a Rat Rhabdomyosarcoma," *Journal of Biological Chemistry* 262 (1987): 3579–3585.

06. R Moro, "Selective Localization of AFP and Serum Albumin within the Sensory Ganglia Cells of Developing Chicken," *Neuroscience Letters* 41 (1983): 253–257.

07. R Moro and J Uriel, "Early Localization of AFP in the Developing Nervous System of the Chicken," *Oncodevelopmental Biology and Medicine* 2 (1981): 391–398.

08. J Uriel et al., "Intracellular Uptake of AFP: A Marker of Neural Differentiation," *Annals of the New York Academy of Sciences* 417 (1983): 321–329.

09. R Moro et al., "In Vivo Uptake of Heterologous AFP and Serum Albumin by Ependymal Cells of Developing Chicken Embryos," *International Journal of Developmental Neuroscience* 2 (1984): 143–148.

10. R Moro, "The AFP Receptor: A Widespread Oncofetal Antigen," in *Biological Activities of Alpha-Fetoprotein*, (Boca Raton, FL: CRC Press, 1989), 119–127.

11. MJ Villacampa et al., "Incorporation of Radiolabeled AFP in the Brain and Other Tissues of the Developing Rat," *Developmental Brain Research* 12 (1984): 77–82.

12. J Uriel et al., "Uptake of Radiolabeled AFP by Mouse Mammary Carcinomas and Its Usefulness in Tumor Scintigraphy," *Cancer Research* 44 (1984): 5314–5319.

13. MJ Villacampa et al., "AFP Receptors in a Human Breast Cancer Cell Line," *Biochemical and Biophysical Research Communications* 122 (1984): 1322–1327.

14. C Esteban, M Geuskens, and J Uriel, "Activation of an Alpha-Fetoprotein (AFP) Receptor Autocrine Loop in HT-29 Human Colon Carcinoma Cells," *International Journal of Cancer* 49 (1991): 425–430.

15. C Esteban et al., "Activation of an Alpha-Fetoprotein/Receptor Pathway in Human Normal and Malignant Peripheral Blood Mononuclear Cells," *Leukemia* 7 (1993): 1807–1816.

16. M Hajeri-Germond et al., "The Uptake of AFP by C1300 Mouse Neuroblastoma Cells," *British Journal of Cancer* 51 (1985): 791–796.

17. R Moro et al., "Monoclonal Antibodies Directed against a Widespread Oncofetal Antigen: The Alpha-Fetoprotein Receptor," *Tumour Biology* 14 (1993): 116–130.

18. R Moro et al., "The Use of Radioiodinated AFP for the Scintigraphic Detection of Mouse Mammary Carcinomas," *Nuclear Medicine Communications* 5 (1984): 5–12.

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