Elevation of Serum Riboflavin Carrier Protein in Breast Cancer


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Abstract
Presently available tumor markers have had a limited clinical impact. Riboflavin carrier protein (RCP) is an estrogen inducible protein that occupies a key position in riboflavin metabolism. Because other vitamin carrier proteins (VCP) have been shown to be overexpressed in patients with malignant disease, we evaluated serum RCP levels in patients with adenocarcinoma of the breast. In this prospective blinded study, patients with breast cancer, benign breast disease, and healthy controls were analyzed for RCP levels. Using a highly sensitive RIA, we observed that serum RCP levels were significantly elevated in women with breast cancer (n = 52) as compared with control subjects (n = 50; 6.06 ± 7.27 ng/ml versus 0.70 ± 0.19 ng/ml (mean ± SD), respectively; P < 0.0001). A serum RCP level of ≥1.0 ng/ml was highly predictive of the presence of breast cancer, detecting 88% of tumors in stages I-II and 100% of tumors in stages III-IV. Overall, this RCP assay has a sensitivity of 92.3%, a specificity of 88%, a positive predictive value of 88.9%, and a negative predictive value of 91.7%. These results show increased serum levels of RCP in breast adenocarcinoma patients and suggest that RCP levels may be useful as a new marker for breast cancer. The positive predictive value in early-stage breast cancer suggests that the RCP assay may be a useful adjunct to present screening technology.

Introduction
Although lung cancer is the leading cause of cancer death in women in the United States, breast cancer remains the leading cause of cancer-related death in women between the ages of 40 and 55 (1). Because early detection, diagnosis, and therapy is associated with greater than 90% survival rate, it is of critical importance that reliable screening techniques be optimized.

Riboflavin and related compounds are essential requirements for cell growth and development. The transport and subsequent deposition of riboflavin in the developing embryo is facilitated by a phosphoglycoprotein called RCP, which binds the protein in a 1:1 molar ratio (2). RCP plays a key role in growth and development. This is underscored by gestational studies in rodents and primates, in which immunological interference of RCP causes an inhibition of riboflavin transport to the developing embryo that results in acute fetal wastage and pregnancy termination (3). In the chicken, the inability to produce RCP is associated with embryonic death in the eggs (4). This vitamin carrier protein has been shown to be an estrogen inducible protein (3).

Studies on the role of RCP in the mammary gland have shown that it is synthesized and secreted into the milk by mammary epithelial cells under estrogen and progesterone control (3).

Because breast cancer is also estrogen-related, and because other VBP like FBP (5) and RBP (6) have been shown to be overexpressed in malignant breast tissue, it was of interest to evaluate RCP dynamics in women with breast cancer. We hypothesized that we would observe an increased synthesis and secretion of RCP by the breast cancer cell, and that this up-regulated synthesis would be reflected by elevated levels of the protein in the blood of women with breast cancer. We further hypothesized that the increased synthesis would be reflected in increased immunohistochemical RCP expression in breast tissue.

Materials and Methods
The protocol and patient consent forms for the study were reviewed and approved by the Institutional Review Board of Louisiana State University, New Orleans, LA. A total of 102 women, including 52 with intact breast tumors and 50 control subjects, were studied. The breast cancer patients were consecutively recruited from the Surgical Oncology clinics at the Louisiana State University Medical Center and included 27 Caucasians and 25 African Americans. The average age of the Caucasian women was 56.5 ± 14.3 years and was similar to that of African American women at 50.5 ± 10.73 (range for the whole population, 27–79 years). Forty-one of the 52 tumors presented in stages I and II (stage I, n = 11; IIa, n = 20; IIb, n = 10). Of the remaining 11, 6 were in stage IIIa, 4 were in stage IIIb, and 1 was in stage IV. In this study, a similar distribution of tumor stages was observed in Caucasian and African American women. Data on tumor stage and hormone receptor profile form part of the clinical protocol and were obtained from the clinical faculty.

Control subjects included 8 women with fibrocystic breast...
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Disease, 10 leukemia cases, and 32 volunteers. The mean age of the control subjects was 45.42 ± 9.26 (range, 29–62 years). Patients and controls were recruited between July 1996 and June 1997. Blood samples from both groups were collected from individuals visiting adjoining Breast and Hematology clinics, and volunteers from among hospital staff at similar times in the afternoon (2–5 p.m.). The specimen was drawn in a serum separator tube (Becton Dickinson 6150, vacutainer system, Rutherford, NJ), assigned a code by the clinical faculty, and transported to the laboratory. The serum separator tubes were centrifuged at 8000 × g for 20 min at 4°C. The separated serum were aliquoted into 750-µl aliquots and stored at −70°C until they were assayed. Serum aliquots were considered as “single use” and were not thawed and refrozen.

Serum RCP levels were measured by a highly sensitive RIA developed in our laboratory. The sensitivity of the assay is 250 pg per tube. It is highly specific for RCP and shows no cross-reactivity with FBP or RBP (Fig. 1). Each sample was assayed in duplicate at two volumes (100 and 200 µl) and the total volume used was 600 µl. The remaining 150 µl from the single-use aliquot was discarded. The assay characteristics include an intra-assay variation of 5% and an interassay variation of 8%. The study was carried out in a blind fashion, with the laboratory personnel being unaware of the clinical details of the blood samples to be analyzed and also of whether the samples were from breast cancer patients or control subjects.

Immunohistochemical Localization of RCP in Breast Cancer Tissue. RCP synthesis by breast cancer cells was studied by immunohistochemical analysis of tissue obtained during surgery. Breast tissue was fixed in 5% formaldehyde and blocked in paraffin. Five-µm sections were subjected to immunohistochemical analysis as described previously (6). Briefly, the procedure consisted of reacting the tissue sections with primary antibody after removing the paraffin and hydrating the tissue. This was followed by biotynylated goat antirabbit γ globulin, avidin peroxidase conjugate, and chromogen-substrate (amino-ethyl carbazole) to develop a bright red color (Zymed Co., Lexington, KY). Nonspecific controls included a slide with preimmune serum substituted for the primary antibody. The sections were counterstained with hematoxylin, mounted, and photographed. Fig. 5, A–C, illustrates the results from those experiments.

Statistical Analysis. Mean serum RCP levels were computed and compared using ANOVA. Median RCP levels were compared using the Mann-Whitney U test. Race distributions were compared using the χ² and Fisher’s exact probability test (7). Histograms of RCP levels and a plot of the nonparametric ROCs were plotted based on the presence or absence of breast cancer using established methods (8). From these plots, a serum RCP cutoff level diagnostic of tumor presence was determined. Tumor presence or absence was next compared with serum RCP levels above and below this cutoff level. The correlation between serum RCP levels and tumor presence was further confirmed by the Likelihood ratio, the χ² statistic, the prevalence adjusted bias adjusted kappa (PABAK) coefficient (8), Cohen κ coefficient, and the odds ratio (9). The sensitivity, the specificity, and the positive and negative predictive values for the RCP assay were computed.

Results
The mean age of women with breast cancer was 53.20 ± 12.49 years (range, 27–79 years). Control subjects tended to be younger and had a significantly lower mean age of 45.42 ± 9.26 years (range, 29–62 years).

In women with intact tumors, mean serum RCP levels were 6.06 ± 7.27 ng/ml, and were significantly higher than those in control subjects (0.70 ± 0.19 ng/ml; P < 0.0001). Median RCP levels were similarly higher than those in control subjects (3.55 ng/ml versus 0.70 ng/ml; P < 0.001; Table 1) Fig. 2 is a scatter graph of RCP levels in breast cancer and control subjects.

Bivariate analyses of data yielded significant differences between cancer patients and controls in all variables except race (Table 2).

On the basis of the frequency histogram for serum RCP levels (Fig. 3) and the ROC analyses (Fig. 4), an appropriate cutoff level for serum RCP levels for breast cancer detection was determined to be 1.0 ng/ml and was used for all subsequent analyses. The area under the ROC curve for serum RCP was computed to be 0.970 (95% confidence interval, 0.943–0.997) which was asymptotically different from 0.50 at P < 0.001.

The likelihood ratio χ² test was used to confirm the efficacy of this cutoff level for breast cancer detection. A highly significant difference (P < 0.0001) was observed in a two-by-two table analysis comparing presence/absence of tumor to RCP levels ± 1.0 ng/ml and <1.0 ng/ml (Table 3).

To further assess the reliability of the RCP cutoff level for tumor presence, the PABAK coefficient was used. The bias index was estimated to be 0.0196 and the prevalence index was estimated to be 0.0392. The PABAK was calculated to be 0.804 and determined to be approximately equal to Cohen’s κ.

The RCP test is, therefore, considered to be highly reliable for breast cancer detection. The assay is highly sensitive with a sensitivity of 92.3%, specificity of 88%, and negative and positive predictive values of 88.9 and 91.7%, respectively.

Serum RCP levels in Caucasian (n = 27) and African American women (n = 25) were 4.80 ± 4.79 and 7.42 ± 9.03 ng/ml, respectively. Although a trend toward higher RCP levels in African American women was noted, these levels were not statistically different. Hormone receptor data were available in 39 women. Table 4 provides the data on stratification of serum

Table 1  Serum RCP levels in women with breast cancer and control subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>SD Error</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td>0.70a</td>
<td>0.19</td>
<td>0.026</td>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>52</td>
<td>6.06a</td>
<td>7.27</td>
<td>1.02</td>
<td>0.70</td>
<td>37.00</td>
</tr>
</tbody>
</table>

a P < 0.0001 median RCP level of 3.55 ng/ml was also significantly (P < 0.001) greater than that in control subjects (0.70 ng/ml).
RCP levels by hormone receptor profiles. The highest serum RCP levels were observed in women whose tumors were estrogen receptor-positive (6.95 ± 8.26 ng/ml; n = 24). In contrast, the lowest serum RCP levels of 4.86 ± 2.56 ng/ml were observed in women whose tumors did not show the presence of estrogen receptors (n = 15). There was no significant difference in serum RCP levels between the two groups. The lack of a significant difference may be ascribed to low sample numbers.

Immunohistochemical analysis of tissue demonstrated increased RCP localization in the cytoplasm of the malignant cells of adenocarcinoma as compared with surrounding tissue (Fig. 5A). These cells were characterized by their increased size, with ample cytoplasm and prominent nuclei with chromatin clumps. The RCP localization was not homogeneous in this cell population, with some cells showing higher intensity of staining. Also the localization within the cytoplasm of individual cells was heterogeneous. However, no particular association with any organelle was noted. In contrast, in nonmalignant tissue, RCP expression was confined to the ductular epithelium (Fig. 5B). Fig. 5C is a reagent control showing no localization of RCP, inasmuch as the primary antiserum was substituted with preimmune serum. The reproducibility of the immunohistochemical results was verified by subjecting additional sections of the same tissue to repeated analysis using the same batch of reagents. Intra-batch variability was also tested by comparing RCP expressions between the two reagent batches. Briefly this was accomplished by comparing the scores assigned (on an ascending scale of 1–5) for factors such as the intensity of staining, the extent of staining, the size of the cell, the size of the nuclei, and the number of nuclei. In all comparisons, be they interbatch or intrabatch, the scores for all of the factors were between 4 and 5, which indicated that the data were highly consistent and reproducible.

**Discussion**

The results of our preliminary study demonstrate increased expression of RCP in malignant breast tissue and elevated serum levels in women with breast cancer. Mean serum levels in these women are substantially higher than those observed during the normal menstrual cycle or during pregnancy (2, 3, 10). At the present time, we do not know whether these increased serum levels are a reflection of increased hepatic production or of increased synthesis of RCP by the breast cancer cells to meet their nutritional requirements. In our study a serum RCP level of $1.0$ ng/ml was able to predict tumor presence accurately, correctly predicting 88% (36 of 41) tumors of stages I and II and 100% (11 of 11) of those in stages III and IV. Five cases of breast cancer (stages I and II) were below the threshold of 1.0 ng/ml (false negative), and six cases without tumor were above the threshold [false positive (Table 3)].

Overall, the RCP test demonstrated a sensitivity of 92.3%, a specificity of 88%, and positive and negative predictive values of 88.9 and 91.7%, respectively. In contrast, a combination of physical examination and mammography has a reported sensitivity of 95%, with a specificity of 51%, and a lower predictive value of 77% (11, 12). A recent study has also indicated that screening mammograms have a false positive rate of 33% (13). In addition, the RCP assay requires only a serum sample, is less expensive, and can be more easily adapted for large scale screening.

Our observations are in contrast with the results of Ramesh Babu and Meenakshi (14), who found a major overlap between normal and cancer subjects. However, their study did not characterize the tumors and could have included large numbers of fibroadenomas, which do not show the extensive localization pattern seen in adenocarcinomas.

Serum RCP levels were higher in African American women (7.42 ± 9.03 ng/ml) as compared with 4.80 ± 4.79
ng/ml in Caucasians. Because of the limited number of cases, at this time we can only speculate as to the significance of this finding.

When serum RCP levels were stratified according to the hormone receptor profiles, the highest RCP levels were observed in women with estrogen receptor-positive tumors. At this time, we are unable to speculate about the significance of these results in view of the limited data available.

The increased expression of RCP in breast adenocarcinoma is similar to elevated expression of other VBPs such as FBP (5) and RBP.5 However, serum levels of these two VBPs were not significantly elevated in breast adenocarcinoma,5 thus

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![Table 3: Two-by-two analyses of RCP levels and tumor presence](image)

<table>
<thead>
<tr>
<th>Serum RCP (ng/ml)</th>
<th>Breast cancer</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1.0 ng/ml</td>
<td>47 (90.38%)</td>
<td>6 (12%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>&lt;1.0 ng/ml</td>
<td>5 (9.62%)</td>
<td>44 (88%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

![Table 4: Serum RCP levels and hormone receptor profiles](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Hormone receptor profile</th>
<th>Serum RCP (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ER⁺ (n = 24)</td>
<td>6.95 ± 8.26</td>
</tr>
<tr>
<td>2</td>
<td>ER⁻ (n = 15)</td>
<td>4.86 ± 2.56</td>
</tr>
<tr>
<td>3</td>
<td>PR⁺ (n = 22)</td>
<td>5.55 ± 5.50</td>
</tr>
<tr>
<td>4</td>
<td>PR⁻ (n = 17)</td>
<td>6.91 ± 8.02</td>
</tr>
</tbody>
</table>

5 Our preliminary unpublished results.
Fig. 5. In A, immunohistochemical localization of RCP in adenocarcinoma of the breast shows extensive cytoplasmic localization. B, in contrast, in fibroadenoma, RCP localization is restricted to the ductular epithelium. C, reagent control showing no RCP localization when primary antiserum was substituted with preimmune serum. ×250.
rendering them unsuitable for use as markers to detect early stages of breast cancer.

In summary, our findings suggest that elevated serum RCP levels could potentially prove to be a reproducible marker for early breast cancer detection. However, this study is a preliminary study and needs to be further validated. Furthermore, the role of RCP in other estrogen-responsive cancers (ovary, cervix, and so forth) needs further study and clarification.

References
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