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RIBOFLAVIN CARRIER PROTEIN: A SERUM AND TISSUE MARKER FOR BREAST CARCINOMA

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We have earlier shown that the estrogen-modulated riboflavin carrier protein (RCP) first isolated from the chicken egg is evolutionarily conserved in mammals and is elaborated by lactating mammary gland as demonstrated with rat mammary epithelial cells in culture and confirmed by isolation of the vitamin carrier from bovine milk. In view of several earlier reports that many milk proteins as well as other estrogen-inducible proteins are up-regulated and secreted into circulation in animal models and in women with neoplastic breast disease, we analyzed serum RCP levels in a double-blind study using a specific radioimmunoassay in pre- and post-menopausal women with clinically diagnosed breast cancer at early and advanced stages of the disease and compared these levels with those in normal age-matched control volunteers. Our data reveal that the serum RCP levels in cycling breast cancer patients are 3- to 4-fold higher ($p < 0.01$) than those in their normal counterparts. This difference in circulatory RCP levels between cancer patients and their age-matched normal counterparts is further magnified to 9- to 11-fold ($p < 0.005$) at the post-menopausal stage. In addition, there seems to be a good correlation between rising RCP levels and disease progression, since significantly higher RCP concentrations ($p < 0.005$) are encountered in patients with advanced metastasizing breast cancer versus those with early disease. Using specific monoclonal antibodies, RCP could be localized immunohistochemically in the cytoplasm of invading neoplastic cells of lobular and ductal carcinomas of the breast, indicating that the malignant cells are probably the source of the elevated serum RCP levels in breast cancer. These findings suggest that measurement of circulatory RCP and the immunohistochemical staining pattern of RCP in biopsy specimens could be exploited as an additional marker in diagnosis/prognosis of breast cancer in women.

Key words: *riboflavin carrier protein; radioimmunoassay; serum; stage-dependent; immunohistochemical localization; pre- and post-menopausal*

Riboflavin carrier (or binding) protein (RCP) is an estrogen-inducible phospho-glycoprotein (Mr 37 K) required in egg-laying vertebrates for yolk deposition of the vitamin to support growth and development of the prospective embryo.[1] The vitamin carrier is evolutionarily conserved in mammals including sub-human primates and humans[2] and plays a pivotal role in embryonic development during gestation. Interference with its function by active or passive immunization results in early embryonic loss and pregnancy curtailment.[3,4] Riboflavin concentration in mammalian milk is several fold higher than in the maternal circulation during lactation,[5] even though the circumferential tight junctions between mammary epithelial cells constitute a barrier for free passage of blood constituents.[6] It was therefore attractive to hypothesize that RCP is elaborated by the lactating mammary gland, an estrogen target tissue, to sequester the vitamin and for secretion into milk for neonatal nutrition. This working hypothesis was amply supported by research in our laboratory,[1,7] which showed that: (*i*) RCP can be detected and assayed in the milk of rodents, cows, bonnet monkeys and humans by using a sensitive and specific radioimmuno assay; (*ii*) it can be bulk purified from bovine milk and exhibits physico-chemical and immunological similarities with its avian counterpart; (*iii*) rat mammary epithelial cells in culture elaborate this protein and (*iv*) estrogen and progesterone modulate its biosynthesis *in vivo* as well as *in vitro*.[7] In view of reports in the literature that expression of genes corresponding to milk proteins and other estrogen-modulated proteins are up-regulated in experimental animal models as well as in women with breast cancer,[8–10] we extended our studies to clinically diagnosed human cancer patients to assess whether RCP is also up-regulated during malignancy and if so, whether a correlation exists between circulatory RCP levels and stage-dependent neoplasia.

In 1991, we reported our preliminary data, which indicated that elevated serum RCP levels could be used as a possible biochemical tumor marker.[11] Meanwhile, a study by Ramesh Babu and Meenakshi,[12] seeking a correlation between estrogen receptor status, but not severity of the disease, and RCP levels in breast cancer patients, was somewhat at variance with our findings. We then undertook an extended phase of our investigation to validate our earlier findings and carried out, in addition, immunohistochemical localization of RCP in breast cancer tissue specimens. When these data were being organized for publication, a similar study based on our previous findings[11] appeared in the literature essentially confirming some of our observations.[13] However, these authors did not attempt to correlate RCP levels with either the stage of neoplasia or menstrual status of the women patients. In this communication, we report on the data obtained from both phases of the investigation, which suggest stage-dependent up-regulation of serum RCP levels in cancer patients.

MATERIAL AND METHODS

Isolation of RCP

RCP was isolated from chicken egg white as described earlier,[14] and the purity of the protein was checked by SDS-PAGE. The protein migrated as a single band and had an apparent molecular weight of 37 K.

Generation of antibodies

Polyclonal antibodies against RCP were raised in rabbits as described.[14] The monoclonal antibodies (MAbs) to chicken RCP used in this investigation have been described earlier and shown to cross-react with purified mammalian, including human, RCP.[15,16]

Serum samples

Serum samples were collected for routine laboratory investigations from 43 breast cancer patients from the Kidwai Memorial Institute of Oncology, Bangalore, India (Study I, 1986/87) and from 47 breast cancer patients from the Bangalore Institute of Oncology, Bangalore, India (Study II, 1995/96). Blood samples were collected from patients at the time of diagnosis or prior to treatment, and sera were separated and stored at −20°C until analysis. Control serum samples were obtained through informed consent from pre-menopausal normal healthy volunteers who had regular menstrual cyclicity (28 ± 4 days); those from post-meno-

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pausal women were obtained through the Health Centre of the Indian Institute of Science, Bangalore.

Radioimmunoassay of serum

RCP was radiolabeled with ^{125}I for use as a tracer by the iodogen method.[17] RCP was quantified in the serum samples by liquid-phase radioimmunoassay (LPRIA) using the double antibody-polyethylene glycol (PEG) precipitation method standardized for assay of human serum samples.[15] Using suitably diluted serum samples from patients, the levels of RCP in the various specimens were measured by LPRIA. Intra- and inter-assay variations were insignificant, and the assay method had a sensitivity range of 10–0.01 ng. Variations in the standards and the samples were in the accepted range of ±5%. Every sample was assayed in duplicate in at least three different RIAs, and for every assay, two calibrator concentrations of 1 ng and 100 pg of RCP were used to normalize the RIA. The study was carried out double blind, and the data on the menarche status of individual patients and the stage of the disease were ascertained from the clinical records retrospectively only after assaying the concentrations of serum RCP. Data analysis was carried out using Student's *t*-test.

In view of the significant (~26%) sequence homology between RCP and human folate binding protein (FBP), the rabbit polyclonal antiserum employed in the RIA was tested for cross-reactivity with FBP and found to be nil even at low FBP dilutions.[16]

Clinical diagnosis

Clinical staging of the disease was carried out by the Union Internationale contre le Cancer tumor size, node involvement, metastasis (UICC-TNM) staging system.

Immunohistochemical localization of RCP in breast cancer biopsy speciments

Localization of RCP in breast carcinoma cells was accomplished by immunohistochemistry[18] employing a mixture of specific murine monoclonal antibodies to chicken RCP. None of these antibodies cross-reacted with the FBP.[16] Tissue specimens obtained from breast cancer patients during surgery were immediately fixed in Bouin's fluid and further processed for embedding in paraffin. Tissue sections (7 μm) were re-hydrated using standard protocols and incubated with specific antibodies. To test for the presence of RCP, a mixture of four specific MAbs raised against RCP was used.[15,19] An anti-mucin-1 MAb (115D8, a kind gift of Dr. J.H.M. Hilgers, Vrije University Hospital, Amsterdam, Netherlands) was used as a positive control to detect malignant cells.[20] Normal mouse serum (NMS) IgG was used as a negative control. After overnight incubation with the primary antibody, the endogenous peroxidase activity was blocked using 0.6% H_2O_2 in methanol, following which the tissue sections were incubated with rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP; Sigma, St. Louis, MO). Binding of the antibodies was visualized on addition of the substrate diaminobenzidine (1mg/ml in PBS containing 0.06% H_2O_2). The sections were counter-stained with hematoxylin, dehydrated, clarified in xylene and mounted in DPX. The sections were observed using a Zeiss microscope with a camera attachment and photographed using Kodak film.

Immunoassay for estrogen and progesterone receptors

Estrogen and progesterone receptors in tissue sections were localized immunohistochemically using the corresponding specific antibodies obtained as part of the kits purchased from Novocastra (New Castle, Tyne, UK). The kits used were NCL-ER-6F1 for estrogen receptors (ER) and NCL-PG-1 for progesterone receptors (PR). The secondary antibodies for the assays were purchased from Lab Vision (Fremont, CA; cat. no. TP-D15-HD). The assays were carried out per the manufacturer's instructions.

RESULTS

The RCP levels were assayed in the serum of breast cancer patients and control subjects using a sensitive radioimmunoassay,[21] further sensitized and validated for quantification of RCP in human serum.[22] In the case of cycling women, we chose to measure circulatory levels of RCP at days 18–20 of the menstrual cycle when they were highest as assessed earlier in the laboratory. Analysis of the data from both studies showed that the mean level of RCP in the serum of normal healthy cycling women ($n = 14$) was 3.8 ng/ml (±0.72, range 2–6 ng). On the other hand, the mean level of circulatory RCP in healthy post-menopausal women ($n = 9$) was 1.7 ng/ml (±0.66, range 1–4 ng). This was anticipated, since RCP is an estrogen-inducible protein and the estrogen stimulus falls precipitously with the onset of menopause. In contrast, in patients with clinically confirmed breast carcinoma, the corresponding values for RCP were markedly higher, with median values of 14.7 ng/ml (±5.25, range 3–50 ng) if they were still cycling ($n = 41$) and 17.0 ng/ml (±4.6, range 3–50 ng) if they were post-menopausal ($n = 49$). The mean RCP levels in cycling women were 3- to 4-fold higher ($p < 0.01$) in patients with breast carcinoma than in normal control subjects of the same age group. The difference in the RCP levels was even more significant in post-menopausal cancer patients, in whom the serum RCP concentrations were 9–11 times higher ($p < 0.005$) than those in their normal counterparts. However, an overlap in serum RCP levels from cycling cancer patients with that of normal subjects was seen, since the values in the normal subjects represented the maximum RCP level reached during the menstrual cycle.

Table I compares the circulatory RCP concentrations in healthy women (both pre- and post-menopausal) with those in patients with clinically confirmed early breast disease as well as those with advanced breast cancer with metastasis. These results were significant. The mean level of RCP in women with early breast disease was 8.4 ng/ml (6.4 and 10.4 ng/ml for Studies I and II, respec-

TABLE I – ANALYSIS OF SERUM RIBOFLAVIN CARRIER PROTEIN (RCP) LEVELS IN BREAST CANCER PATIENTS (BOTH EARLY AND ADVANCED METASTATIC CANCER)

	Serum RCP levels (ng/ml)		
	Mean ± SEM	Range	*p*-Value
Study I (1986/87)			
Normal women			
Cycling and postmenopausal ($n = 14$)	3.0 ± 0.48	1–6	
Breast cancer patients			
Early ($n = 13$)	6.4 ± 1.2	2–8	$<0.005^1$
Advanced, with metastasis ($n = 30$)	21.8 ± 2.77	8–50	$<0.0005^2$
Study II			
Normal women			
Cycling and postmenopausal ($n = 9$)	2.6 ± 1.02	1–5	
Breast cancer patients			
Early ($n = 21$)	10.4 ± 5.24	3–22	$<0.005^1$
Advanced, with metastasis ($n = 26$)	19.1 ± 4.87	10–31	$<0.005^2$

[1]In comparison with normal women.–[2]In comparison with patients with early breast disease.

tively) as against 2.8 ng/ml (3.0 and 2.6 ng/ml for Studies I and II, respectively) in normal women. Here again the elevations in RCP levels are approximately 3- to 4-fold ($p < 0.005$). However, the most striking difference was evident when the RCP levels between normal subjects (2.8 ng/ml) and those with advanced breast cancer with metastasis were compared. The mean concentration of serum RCP in these patients was 20.4 ng/ml (21.8 and 19.1 ng/ml for Studies I and II, respectively), which was ~7 times higher ($p < 0.0005$) than the corresponding serum RCP values encountered in age-matched control subjects. These observations clearly reveal that patients with early breast carcinoma harbor higher levels of serum RCP compared with normal disease-free women and that these levels are further elevated in patients suffering from advanced breast cancer with metastasis.

Clinical evaluation of the study subjects was carried out using the UICC-TNM staging system. It was found that levels of serum RCP exhibited a significant correlation with the stage of the disease and the levels were found to be higher in patients with advanced disease.

At this stage it was important to ascertain whether the malignant breast tissue accounts for stage-dependent elevated serum RCP levels in these carcinoma patients. Using the technique of immunohistochemistry, RCP could be localized in cancer tissue using a pool of anti-RCP-specific MAbs. To confirm neoplastic nature of the cells in the tissue samples, a MAb (115D8, which specifically reacts with polymorphic epithelial mucin-1 [MUC1]) was used as a positive control[23] to identify the cancer cells. NMS IgG was used as a negative control. Tissue samples from six breast cancer patients were analyzed and the immunohistochemical staining pattern obtained with the MAb 115D8, anti-RCP MAbs and NMS IgG for tissues from three of the specimens is shown in Figure 1. All the breast cancer tissue specimens exhibited intense staining with the anti-MUC1 MAb 115D8, as expected. The breast cancer tissues also showed positive staining for the presence of RCP although the staining pattern varied. Whereas the MUC1 specific antibody staining was mostly membrane bound, the staining pattern with the anti-RCP MAbs exhibited different distribution patterns depending on whether the cancer was a lobular or infiltrating ductal carcinoma, or whether the cancerous tissue had an acute lymphoid response. In lobular carcinomas (specimen 174/95) the localization of RCP was intra-cytoplasmic as well as peri-nuclear, whereas in the case of infiltrating ductal carcinoma (specimen 57/95) RCP staining was predominantly intra-cytoplasmic. Sample 101/95 was an infiltrating ductal carcinoma with an acute lymphoid response. In this sample most of the cells were inflammatory cells and hence did not stain for RCP. However, the cancer cells, which were very few in number, did exhibit both intra-cytoplasmic and peri-nuclear staining (very faint in Fig. 1). The staining pattern was specific to RCP as none of the samples showed positive staining with NMS IgG. Pathological details on the samples and type of staining pattern observed are collated in Table II.

Analysis of the samples for ER and PR was carried out by immunohistochemistry. Only one sample (174/95) stained positive for ER (40%). However, none of the specimens showed staining for PR. The results are included in Table II.

DISCUSSION

Clinical and experimental evidence has established the central role of estrogen in the genesis of breast cancer. About 85% of all breast cancers are estrogen dependent, and these require the hormone for continued growth and differentiation.[24] Many studies have also shown that ER levels are also significantly higher in malignant breast epithelium compared with normal mammary tissue.[25] Since RCP levels are also regulated by estrogen under physiological conditions, we sought to investigate whether there was a marked change in RCP levels in patients with breast cancer compared with their normal age-related counterparts. Hence the primary aim of our investigations was to compare the serum RCP levels of individuals who had clinically confirmed breast cancer with those of normal healthy individuals. Additionally, it was of interest to investigate whether circulating RCP levels exhibit any correlation with the disease stage, node involvement and metastasis. We anticipated that if elevated RCP levels reflect the severity

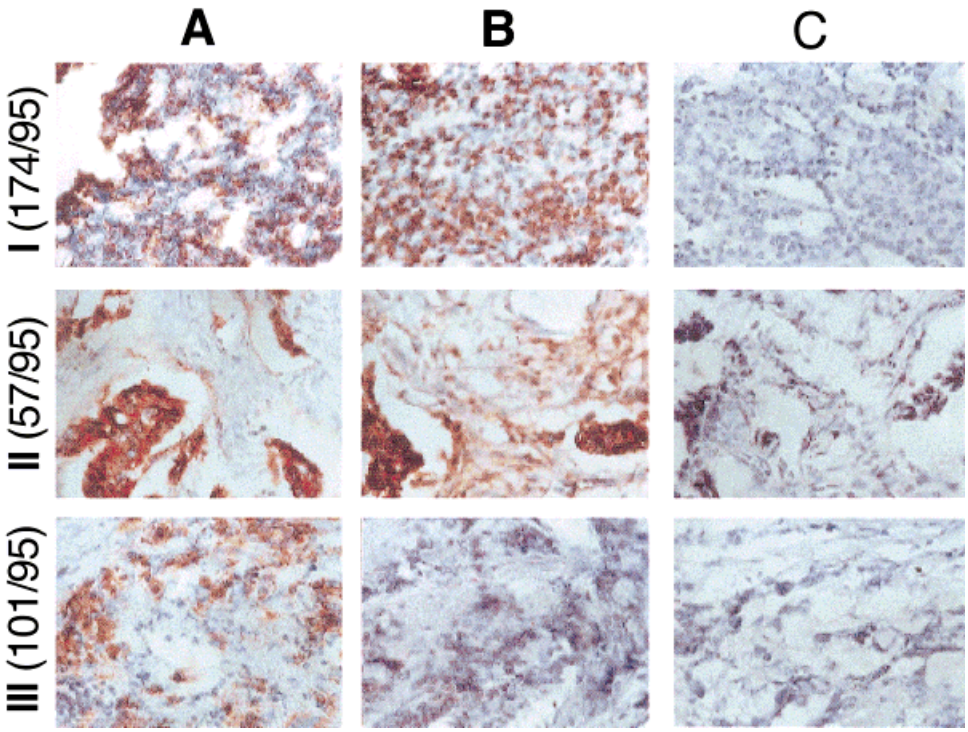

FIGURE 1 – Immunohistochemical staining pattern of specimens from three breast cancer patients. I, sample no. 174/95 (lobular carcinoma); II, sample no. 57/95 (infiltrating ductal carcinoma); and III, sample no. 101/95 (infiltrating ductal carcinoma with an acute lymphoid response) seen with anti-mucin MAb 115D8 (*a*), anti-RCP MAbs (*b*) and NMS IgG (*c*). Magnification ×200.

TABLE II – PATHOLOGY, STAINING PATTERNS AND RECEPTOR STATUS OF THE SAMPLES

Sample no.	Type of cancer	Staining pattern		ER status	PR status
		With Mab 115D8	With RCP Mabs		
174/95	Lobular carcinoma of the breast (cribiform pattern seen)	Membrane-bound and intracytoplasmic staining	Intracytoplasmic staining and perinuclear staining	Positive (40%)	Negative
157/95	Infiltrating ductal carcinoma	Membrane-bound staining	Intracytoplasmic staining; no perinuclear staining	Negative	Negative
121/95	Infiltrating ductal carcinoma	Membrane-bound staining	Intracytoplasmic staining; no perinuclear staining	Negative	Negative
105/95	Lobular carcinoma of the breast	Uniform membrane-bound and intracytoplasmic staining	Intracytoplasmic and perinuclear staining in 50% of cancer cells	Negative	Negative
101/95	Infiltrating ductal carcinoma of the breast showing an acute lymphoid response	Tumour cells show membrane-bound and intracytoplasmic staining, Lymphocytes are negative	Intracytoplasmic and perinuclear staining in cancer cells; most of the cells are inflammatory cells, which do not show staining	Negative	Negative
57/95	Infiltrating ductal carcinoma with abundant desmoplasia (fibrous tissue proliferation)	Very intense membrane-bound staining	Very dense intracytoplasmic staining	Negative	Negative

of the malignant disease, the measurement of this specific protein could serve as a non-invasive marker for the presence of breast cancer, to monitor the response to therapy and to assist prognosis. Furthermore, to ascertain whether the enhanced RCP in the circulation originates directly from malignant cells due to destruction of original tissue architecture, we used immunohistochemical techniques and specific MAbs to examine the pattern of distribution of RCP in biopsy tissue specimens from breast cancer patients.

In the present study, the serum RCP concentrations measured in breast cancer patients were significantly higher than those encountered in control individuals of the same age group and were dependent on the stage of the disease. In normal women, RCP levels in circulation vary in concert with estrogen levels depending on the stage of the menstrual cycle. It has been reported that RCP levels in serum were highest 3–4 days after the ovulatory surge of estrogen.[22] These values represent the highest levels of RCP reached in the circulation, and we chose to use them for comparison with those from cancer patients. In a recent publication by Rao *et al.*,[13] an average value of 0.7 ng/ml RCP in the circulation of normal women was reported. The authors were apparently unaware of hormonal status influencing circulatory RCP levels during the menstrual cycle and hence the values reported may be lower than expected. In the present study, as anticipated, we encountered relatively low levels of RCP in post-menopausal women. In view of this situation, it is not surprising that the difference in RCP levels between patients and normal individuals is much more striking in post-menopausal women. Thus, whereas the patients with early breast disease exhibit significantly higher levels of RCP (3- to 4-fold) compared with healthy controls, this difference is strikingly magnified (>7-fold) in patients with advanced breast cancer with metastasis.

In attempts to ascertain the likely tissue source of the elevated RCP in breast cancer, it was indeed very encouraging to find intense immunostaining of RCP in breast cancer tissues when specific anti-RCP MAbs were employed for detection. All six breast cancer tissue samples studied showed positive staining for RCP as well as for the cancer marker MUC1. However, there appear to be significant differences in the pattern of immunostaining of RCP compared with MUCI in that RCP is mostly localized to the cytoplasm and is occasionally peri-nuclear (probably representing the nascent glycoprotein undergoing modification in the Golgi complex), whereas MUCI is mostly confined to membranes of cancer cells.[26,27] In normal breast tissue, MUC1 is expressed at the apical surface of glandular epithelium. However, polarization is lost in carcinomas, where it is expressed on the entire cell surface.[28] Moreover, MUC1 is overexpressed and deficiently glycosylated in adenocarcinomas and gains access to the circulation in patients.[23] Levels of MUC1 in blood reflect recurrence and progression of the disease.[23,29] Based on these data, it is attractive to speculate that up-regulated RCP production by invading cancer, cells coupled with carcinoma-induced destruction of original tissue architecture, leads to RCP gaining entry into the stroma, lymph and blood vessels and hence reflects the stage-dependent severity of malignant condition.

Breast carcinoma is the most common form of cancer in middle-aged women and is a leading cause of death.[30] It is the second most common form of cancer in women in the Indian subcontinent according to the population-based registry data (NCRP 94). As with all neoplastic diseases, early detection is of vital importance in the eventual prognosis of the disease. The availability of many reliable screening methods significantly improves the chances of detection, which in turn helps in the immediate commencement of therapy. Many prognostic factors, both biological and non-biological, are used in clinical practice to identify high-risk individuals and to plan aggressive multi-modality treatment protocols to achieve better long-term results. However, since no single absolute marker has been found so far it, would be advisable to use a panel of markers. The ER has also been found to be a very useful tool for prognosis.[31,32] Many studies have shown that malignant breast tissues express a higher level of ER than normal breast tissue,[25] and such patients receive objective benefit from hormonal therapy. However, approximately 30% of ER+ tumors fail to respond to endocrine therapy, which could be due to tumor heterogeneity or non-functionality of the hormone receptors.[33]

In this study we have evaluated the possibility of using RCP as a marker for breast cancer. Since the protein is estrogen inducible and since it is well established that up to 85% of breast cancers are estrogen dependent, it was considered as a possible candidate. There are several advantages in using RCP as a possible marker for breast cancer. Its levels can be easily monitored in serum by a standard sensitive LPRIA, which is very easy to carry out routinely with a large number of serum samples; this assay is also non-invasive and hence could be used to study disease progression over a period of time. RCP is regulated by estrogen under physiological conditions;[1] therefore it was surprising to find that five of the six breast cancer tissue samples investigated were negative for estrogen receptors by the immunochemical assay kit used. However, some degree of correlation between RCP levels and estrogen receptor status has been reported by Ramesh Babu and Meenakshi[12] in a small sample size. There is a possibility, however, that during carcinogenesis, the regulation of RCP synthesis could become estrogen independent. A similar situation was encountered

with cathepsin D, another independent breast cancer marker in which the synthesis of the protein is de-linked from regulation by estrogen under certain conditions of malignancy. It has been shown that in both the estrogen receptor-positive and -negative breast cancer cell lines the mRNA coding for pro-cathepsin D is over-expressed.[34] This is further supported by the observation that markedly enhanced levels of serum RCP were found in post-menopausal women with breast carcinoma, whereas their healthy counterparts exhibited very low levels of the protein. The elevated levels of RCP in circulation in breast cancer patients appear to originate from the cancer tissue since patients with metastasis showed markedly higher levels of RCP than patients with localized tumor, the tumor cell burden being higher during metastasis. We have earlier observed that immunochemical distribution of RCP in normal breast tissue is almost exclusively localized to ductal epithelium, whereas, in the present study with the cancer tissues, this pattern seems to have altered significantly with respect to RCP staining associated with malignancy. It is conceivable therefore that immunohistochemical localization of RCP using fine needle biopsy specimens maybe useful in early diagnosis of *in situ* carcinomas. The ability to immunolocalize RCP in paraffin-embedded tissue sections should make it possible to obtain data on RCP in retrospective series of patients with breast cancer in relation to clinical diagnosis/prognosis. Further research efforts on these lines should be useful in exploring RCP as a biochemical marker for malignancies of breast. The above preliminary data were obtained retrospectively with a limited population of cancer patients, and they await confirmation in a larger number of patients. It may be useful to assess levels of RCP in the same patient over a long period of time starting from the stage of initial diagnosis through any treatment regimes since it is likely that assay of serum RCP may become useful as a marker to assess prognosis and follow-up therapy and to classify risk factors in breast cancer patients.

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