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SHORT REPORT

ANALYSIS OF THE LEVEL OF mRNA EXPRESSION OF THE MEMBRANE REGULATORS OF COMPLEMENT, CD59, CD55 AND CD46, IN BREAST CANCER

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We have examined the relative mRNA expression of the complement (C) regulatory proteins CD59, CD55 and CD46 in RNA isolated from 50 primary breast cancer specimens using a semiquantitative RT-PCR approach. Having normalized the mRNA expression levels of the C regulators relative to actin, we subsequently correlated their expression with estrogen receptor (ER) and various clinical, pathologic and biochemical features of the disease. CD59 and CD46 were detected in all clinical biopsies, while CD55 mRNA was detected in the majority of samples. The comparative levels of expression between the 3 regulators analyzed, using Spearman rank correlation test, revealed a significant association ($p = 0.01$; $r = 0.36$) between CD46 and CD59. CD46 exhibited the most striking pattern of association, with increased levels of expression being associated with ER-positive samples and lower levels of expression associated with a loss of differentiation and epidermal growth factor receptor positivity. Application of Spearman rank correlation test revealed CD46 expression was significantly associated with expression of ER at the level of protein ($p = 0.031$; $r = 0.31$) and mRNA ($p < 0.001$; $r = 0.52$). CD46 expression also correlated with insulin-like growth factor receptor-positive samples using Spearman rank correlation test ($p = 0.016$; $r = 0.34$), but negatively associated with tumor samples either exhibiting histologic grade 3 when compared to grades 1 or 2 or displaying elevated levels of inflammatory cell infiltrate. Immunohistochemical analysis of a limited series ($n = 8$) of paraffin-embedded breast cancers indicated that the level of CD46 protein expression directly associates with that of the mRNA and, where prominent, is localized in the tumor epithelial cell population, including at the plasma membrane. These data provide new information on expression of these important regulators in breast cancer and suggest that CD46 should be evaluated as a novel prognostic indicator.

Key words: *breast; tumor; complement; CD59; CD55; CD46*

The universal acceptance of immunotherapy as an effective cancer treatment strategy remains unfulfilled partly because of a lack of an effective humoral response to tumor cell antigens. This may be partly explained by the presence of both soluble and membrane-bound complement regulatory proteins (CRPs) that serve to control immune-mediated cell death. Tumor cells, like normal cells, are frequently resistant to complement (C), largely because of the presence of a number of membrane-bound CRPs that either limit the formation of the C3/C5 convertase enzymes or inhibit the formation of the membrane attack complex.[1]

The principal membrane-bound CRPs include CD46 (membrane cofactor protein; MCP), CD55 (decay-accelerating factor; DAF) and CD59. These protective proteins are broadly expressed on a wide variety of tissues and cell surfaces, including epithelial, endothelial and circulatory cells, and act as physiologic brakes to the C cascade amplification process. CD46, CD55 and CD59 have been shown to be expressed on a number of solid tumors and their associated cell lines and it is becoming increasingly apparent that the level of expression of these 3 proteins in malignant tissue is at least equivalent to, or sometimes greater than, that seen in the normal surrounding tissue.[2–4] Consequentially, the increased C resistance conferred by these membrane-bound CRPs has been proposed as a mechanism that facilitates the survival of the tumor or the metastasizing tumor cell when it enters circulation.[5–7] Even though these proteins are ubiquitously expressed, there are observed variations in the relative levels of protein expression between the 3 membrane-bound regulators within normal and tumor tissues. Niehans *et al.*[8] have shown that whereas colon carcinoma stained for the presence of CD46, CD59 and CD55, ductal carcinomas of the breast expressed different combinations of all 3 inhibitors. The functional importance of these proteins as C regulators in tumor cells has been eloquently addressed by anti-CRP antibody-blocking experiments. For example, effective neutralization of CRPs including CD59,[9] CD55 or CD46 specifically augments the lysis of breast carcinoma cells when attacked by homologous C.[10]

Whereas CRP expression at the protein level in breast cancer tumors and cell lines has been examined, very little information is available regarding the levels of expression of CRP mRNA within breast tumor tissue and relationship to clinicopathologic features of the disease. Therefore, in an effort to understand the importance of the membrane-bound CRPs in breast cancer, we have examined the mRNA expression levels of 3 membrane CRPs, CD59, CD55 and CD46, in 50 breast cancer specimens using a semiquantitative PCR approach. The research objectives were to determine the levels of CRP mRNA expression relative to actin and to correlate relative expression values between the 3 CRPs, estrogen receptor (ER) status and other clinical, pathologic and biochemical features of the disease. The data provide a greater understanding of the importance of the level of CRP mRNA expression in relation to other known breast cancer parameters and permit us to speculate on the use of anti-CRP breast cancer treatment strategies.

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MATERIAL AND METHODS

Patients and tissues

The patients in this study presented with primary operable breast cancer to a single surgeon (J.F.R.) at the Nottingham City Hospital (Nottingham, U.K.). Primary breast cancer specimens from 50 patients, presenting for surgery at the City Hospital, were made available for this investigation. Parallel histologic grade of malignancy, menopausal status, lymph node stage and patient survival data, together with information on ER mRNA and protein, EGFR protein and IGF-IR mRNA,[11] were also available. In all cases, representative excised tissue samples were snap-frozen in liquid nitrogen and stored at −70°C solely for RNA analysis.

Reverse transcription-PCR amplification

Total RNA was isolated from the frozen breast tumor specimens together with comparable samples from 2 human breast cancer cell lines (MDA 231, MCF-7) and reverse-transcribed according to published methods.[12,13] Briefly, 1 μg of total RNA, previously denatured (95°C, 5 min) and ice-quenched (4°C, 5 min), from each sample was added to an ice-cold mixture (total volume, 20 μl) containing dNTPs (625 μM), dithiothreitol (10 mM), random hexanucleotides (pd[N]$_6$, 100 pmol; Pharmacia, Uppsala, Sweden), RNasin (25 U; Promega, Madison, WI), Rnase/DNase free water and 1 μl MMLV reverse transcriptase (200 U; Gibco-BRL, Gaithersburg, MD) in reverse transcription (RT) buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.002% w/v gelatin). Reaction mixture was heated to room temperature (20°C) for 10 min, reverse-transcribed 40 min at 42°C; reaction was finally terminated by heating to 95°C (RT mixture). Negative control (lacking RNA) was also included to check for contamination.

Primers for actin, CD59, CD55 and CD46 were all designed from mRNA sequence data using the Oligo 6 primer analysis design software (Molecular Biology Insights, Cascade, CO) and spanned individual exons, thus ensuring minimal genomic DNA amplification. The forward primers synthesized are labeled according to the first-base position in the coding strand (5′-U) sequence and reverse primers are labeled according to the position of the first complementary base (3′-L). The size of each PCR product generated is indicated, as is the Genbank accession number: actin-835U, 5′-CCTTCCTGGGCATGGAGTCCT-3′, 201 bp (X00351); actin-1017L, 5′-GGAGCAATGATCTTGATCTT -3′; CD59-64U, 5′-ATGGGAATCCAAGGAGGGT-3′, 380 bp (X16447); CD59-426L, 5′-ATGAAGGCTCCAGGCTGCT-3′; CD46-310U, 5′-GCTACCTGTCTCAGATGACG-3′, 411 bp (Y00651); CD46-701L, 5′-ACCACTTTACACTCTGGAGC-3′; CD55-1044U, 5′-GCAACACGGAGTACACCTGT-3′, 361 bp (NM000574); CD55-1383L, 5′-GCTAAGAATGTGATTC-CAGG-3′.

PCR amplification

Multiplex PCR conditions for each of the CRP primer pairs with the reference primer pair for actin were initially optimized using RT mixture (cDNA) derived from RNA isolated from MCF-7 and MDA-231 breast cancer cell lines and additional tumor RNA samples.[14,15] The individual CRP genes were coamplified with actin using the same buffer conditions for PCR amplification. Briefly, multiplex PCR was performed by adding 1 μl of RT mixture from cell lines or tumor samples to an ice-cold PCR buffer [67 mM Tris-HCL, pH 8.8 (at 25°C), 16 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.1% Tween-20, containing 0.1 mM dNTPs] containing each of a pair of CRP oligonucleotide primers (CD59, 10 pmol; CD55, 10 pmol; CD46, 10 pmol) with each of the respective pair of reference actin primers (4 pmol; 2 pmol; 2 pmol) and 0.125 U of Biotaq (Bioline, London, U.K.) to a total volume of 25 μl. Individual breast cancer samples were amplified for the presence of CRP and actin. Sample tubes were carefully added to a previously heated (90°C) PCR block and cycling was initiated. The cycling parameters comprised an initial denaturation temperature of 95°C for 3 min, an amplification step comprising 94°C for 30 sec, 55°C for 30 sec (ramp rate, 1°C/sec) and 72°C for 30 sec for a total of 25 (CD59), 26 (CD46) or 30 (CD55) cycles appropriate to the CRP-amplified product, and a final single extension cycle of 72°C for 5 min. Aliquots (8 μl) of amplified DNA products were resolved using 3% agarose gel electrophoresis, stained with ethidium bromide, washed, viewed and the fluorescence of the CRP and actin PCR fragments densitometrically quantified using a Bio-Rad Gel 1000 Documentation system and software. CRP densitometric values were normalized relative to actin and subtracted for background fluoresence. PCR products were also cloned and sequenced to confirm specific gene product amplification.

Immunohistochemistry

A limited number ($n = 8$) of formalin-fixed, paraffin-embedded primary breast tumors matched for CD46 RNA analysis were available to examine CD46 protein expression immunohistochemically. CD46 protein detection involved the use of an affinity-purified rabbit polyclonal anti-CD46 antibody, produced and validated in house and employed immunohistochemically essentially as previously described.[16] Briefly, 5 micron sections of each sample were exposed to microwave treatment for 30 min in 0.2% citric acid buffer (pH 6.0) for antigen retrieval, washed in phosphate-buffered saline (PBS), then incubated with 1.33 μg/ml rabbit polyclonal anti-CD46 primary antibody for 16 hr at 4°C. Matched control sections were incubated either with an irrelevant rabbit anti-CD35 antibody or with 1.33 μg/ml anti-CD46 primary antibody previously preincubated at 4°C for 1 hr with 100-fold excess purified CD46 protein. After PBS washing, slides were incubated with 1/100 secondary goat antirabbit IgG-horseradish peroxidase-labeled antibody (Bio-Rad, Richmond, CA) at room temperature for 1 hr. Diaminobenzidine tetrahydrochloride (0.05%; Sigma, Dorset, U.K.) with hydrogen peroxide (0.005%; Sigma) was then applied for 10 min, finally counterstaining and mounting slides. Tumor cell immunostaining was viewed with a light microscope and photographed.

Statistical analysis

Comparative analysis of densitometric data was performed using the SPSS version 10 statistical analysis package (SPSS, Chicago, IL) using a 2-sided Mann-Whitney U-test, together with a Spearman rank correlation test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Assay development

A pilot study of RT-PCR cycle number compared to PCR product revealed that all 3 CRP mRNA species could readily be detected in the breast cancer cell lines MCF-7 and MDA-231 at cycle numbers 21, 24 and 27 for CD59, CD46 and CD55, respectively (data not shown). CD59 and CD46 could be detected in breast cancer specimens at cycle numbers of 25 and 26, respectively, and were within the linear range of the amplification profile at these cycle numbers. An additional 5 cycles were necessary to identify CD55 mRNA within the linear amplification portion of the reaction profile. Using these differential assay conditions, all tumors were CD59 and CD46 mRNA-positive, whereas 46/50 (92%) were CD55 mRNA-positive. Representative examples of the PCR products of CD59, CD46 and CD55 are shown in Figure 1. Following correction of CRP mRNA relative to actin, the relative levels of expression of CD59, CD55 and CD46 showed a range of 0.22–1.91, 0–2.4 and 0.6–22.7, respectively, with median values of 0.79, 0.86 and 4.13 being recorded, respectively. Comparison of all the patient samples revealed a positive association ($p = 0.01$; $r = 0.359$) using Spearman rank correlation analysis between the CD46 and CD59 mRNA expression levels in tumors.

Clinical and biologic associations

Of the CRPs examined, only CD46 levels showed any obvious association with the biologic and pathologic features of the breast

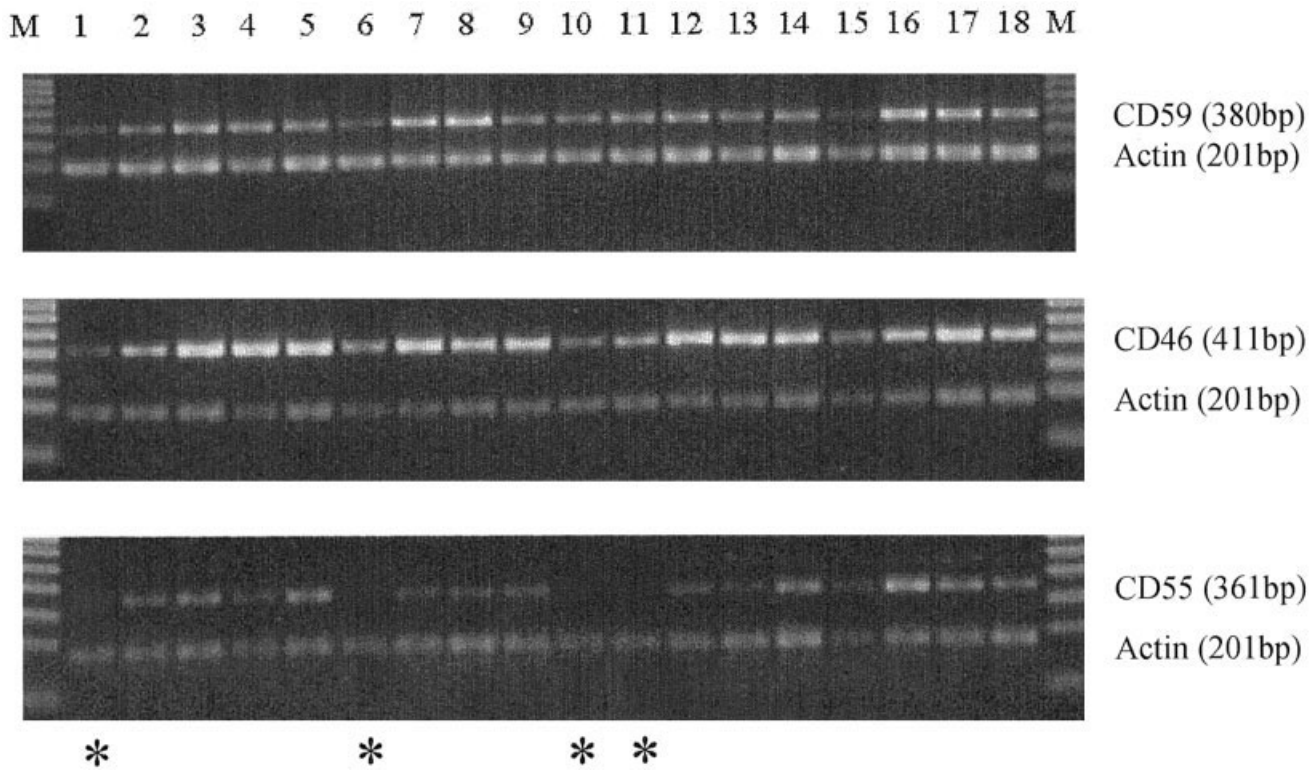

FIGURE 1 – Differential expression of the complement regulators CD59 (380 bp), CD46 (411 bp) and CD55 (361 bp; lanes 1–18, from top to bottom, respectively), together with the coamplified actin housekeeping gene (201 bp) is shown for 18 out of a total of 50 breast tumor samples using specific primer concentrations and cycle numbers as indicated in text. Four of the tumors negative for CD55 (asterisk) together with 100 bp DNA molecular weight ladder (M) are indicated.

TABLE I – CORRELATION OF RELATIVE LEVEL (TO ACTIN) OF EXPRESSION OF CD46 mRNA IN BREAST CANCER BIOPSIES WITH CLINICAL, PATHOLOGICAL AND BIOCHEMICAL FEATURES OF THE DISEASE

Clinical parameter	*n*	CD46 range	CD46 median	*p*-value
ER mRNA-negative (ER mRNA < 0.85)	25	0.56–7.24	2.9	<0.001
ER mRNA-positive (ER mRNA ≥ 0.85)	25	2.32–22.7	5.1	
EGFR-negative (EGFR $= 0$)	24	1–22.7	5.2	0.011
EGFR-positive (EGFR > 0)	23	0.56–11.44	3.5	
IGF-IR mRNA-negative (IGF-IR mRNA < 0.58)	24	0.56–11.44	3.7	0.048
IGF-IR mRNA-positive (IGF-IR mRNA ≥ 0.58)	26	1.95–22.7	4.9	
Histologic grade 1/2	17	1–11.44	5.1	0.029
Histologic grade 3	21	0.56–22.7	3.7	

Significant correlation of the expression of CD46 with the presence (positive) or absence (negative) of ERα mRNA, IGF-IR mRNA, EGFR protein, or histologic grade subpopulations is shown. The number in parentheses specifies parameter cutoff values corresponding to the median, associated with each group. n, the number of tumor samples associated within each group and the minimum and maximal values (range) of CD46 expression are indicated. The median and significant correlation (p) values is represented.

cancers examined. In this series, CD46 levels were significantly elevated in ERα mRNA-positive (Table I; $p < 0.001$) specimens. A similar association was observed for the level of CD46 expression with insulin-like growth factor receptor (IGF-IR) mRNA (Table I; $p = 0.048$). These associations were also retained when the absolute levels of ER and IGF-IR were analyzed with CD46 expression status [Spearman analysis, $p < 0.001$, $r = 0.52$ (Fig. 2) and $p = 0.016$, $r = 0.34$ (data not shown)]. In contrast, CD46 levels were negatively associated with the epidermal growth factor receptor (EGFR) protein status of the tumors (Table I; $p = 0.011$), a relationship that was also maintained in a Spearman analysis ($p = 0.005$; $r = -0.41$; data not shown). Identically, CD46 expression levels were also negatively associated with the histologic grade of malignancy of the tumors (Table I; $p = 0.029$) when the CD46 values of grade 1 or 2 tumors were compared to the values obtained in grade 3 tumors. The level of expression of CD46 was also negatively associated with the level of infiltrating lymphocytes (Spearman analysis, $p = 0.009$; $r = -0.039$; data not shown). No association was observed between the level of expression of CD46 mRNA with the cellular proliferation indicator Ki67, menopausal status, lymph node staging of the disease and patient survival (data not shown).

Immunohistochemical analysis of the limited available series of paraffin-embedded primary breast cancers for CD46 protein revealed that this protein was detectable in all the samples examined, as indicated by the presence of any degree of specific immunostain. Importantly, however, the level of protein expression, as measured by the intensity and prevalence of immunostaining, was noted to be heterogeneous between tumors. Thus, strong immunostaining was apparent in the tumor epithelial cells comprising samples exhibiting elevated CD46 mRNA expression (Fig. 3*a* and

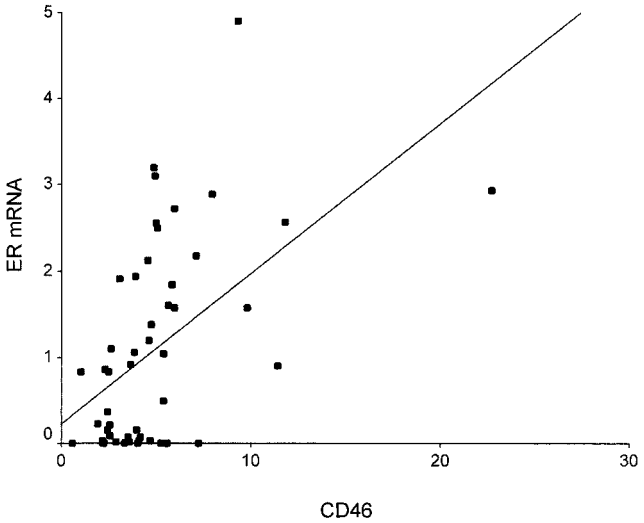

FIGURE 2 – Correlation of the relative (to actin) levels of mRNA expression, as measured by densitometry, of CD46 and ERα in breast cancer biopsies ($n = 50$; $p < 0.001$; $r = 0.52$). The linear relationship between 2 parameters is indicated.

b). Staining was prominent at the tumor epithelial cell membranes and heterogeneously within their cytoplasm. In contrast, CD46 immunostaining was barely detectable within samples where a lower relative value of CD46 mRNA expression had been recorded (Fig. 3*c*). Subsequent Spearman analysis of CD46 protein expression in this small series further indicated a positive association with the level of the mRNA ($p = 0.031$; $r = 0.755$). Matched control sections incubated with an irrelevant anti-CD35 antibody confirmed the specificity of the immunostaining observed, as did preincubation of the anti-CD46 primary antibody with purified CD46 protein where a marked loss in the intensity of CD46 staining was apparent in sections incubated in the presence of the immunogen (data not shown).

DISCUSSION

Although the existence of C activation products within tumor specimens derived from breast,[8,17,18] kidney[19] and thyroid[20] tissues has been established, the involvement and regulation of the C system in tumor maintenance has yet to be fully explored.

Analysis of the levels of expressed mRNA of the CRP has been previously reported for some of human tumors and tumor cell lines.[3,21–24] However, there has been no attempt to correlate CRP mRNA expression to other associated disease parameters. In an effort to address this deficiency, we have measured the levels of expression of CRP mRNA relative to actin in a large panel of well-characterized primary breast tumor samples and correlated the results to other known biologic parameters.

First, we established optimum conditions for semiquantitative RT-PCR of each of the CRPs. The differences in the optimal PCR cycle linear amplification threshold values between CD59 (25 cycles), CD46 (26 cycles) and CD55 (30 cycles) in breast cancer tumor samples suggest a relative difference in mRNA abundance between the 3 regulators of the order CD59 $\geq$ CD46 $>>$ CD55. Interestingly, Thorsteinsson *et al.*[25] reported, using immunohistochemical analysis, that CD46 and CD59 proteins were expressed in the majority of ductal breast carcinomas assayed, whereas CD55 expression was not detected. Similarly, Niehans *et al.*[8] reported that breast tumor cell membrane staining pattern for CD55 could not be detected, whereas CD59 and CD46 were present in 30% and 100%, respectively, of breast tumor specimens analyzed. Such immunohistochemical data, however, fail to take into account the possibility of increased protein turnover or the presence of soluble CRP forms, normally found in a variety of biologic fluids. Seya *et al.*[26] found elevated levels of soluble CD46 in the majority of serum samples from cancer patients. Likewise, melanoma cells are capable of producing a soluble CD59 moiety[27] and a soluble form of CD55 has been reported to be synthesized from HeLa cells.[28] Indeed, large quantities of CD59 and CD55 have been detected in breast tumor tissue stroma[8] and suggested to be soluble tumor-derived forms, although this suggestion remains unsubstantiated. Analysis of the relative levels CRP of mRNA expression may therefore prove to be a more appropriate quantitative test to obtain valid correlation since it circumvents the inherent problems noted above associated with the assessment of CRP moieties.

The positive correlation of the level of CD46 mRNA with ER mRNA ($p < 0.001$) and protein ($p = 0.031$) suggests that the control of expression of both proteins may be linked by a common factor. Although there is no evidence that the expression of CD46 is regulated by estrogen or other steroids, human CD46 has been shown by immunohistochemistry to be diffusely and strongly expressed within normal epithelia and vascular endothelial tissues of the female reproductive tract,[29] suggesting a relationship between CD46 expression and the female steroid hormones, although this hypothesis remains to be determined.

The observed inverse correlation of CD46 with EGFR ($p = 0.005$) is anticipated from the positive association with ER since the existence of an inverse correlation between ER and EGFR in breast tumor tissue has been reported.[30–32] This anticipated association can be extended to the inverse correlation of CD46 with histologic tumor grade since there is reduced expression or absence of ER in poorly differentiated grade 3 tumors when compared to grade 1 or 2 tumors.[33] The level of CD46 expression observed within the breast tumors in this study does not appear to be attributable to the presence of tumor infiltrating lymphocytes because of the observed inverse correlation with the level of infiltrate ($p = 0.009$). Our PCR data, however, contradict a published immunohistochemical analysis of CD46 expression in human breast carcinomas, which revealed a strong diffuse staining pattern that increased with tumor grade.[2] In an effort to address this disparity, we compared the expression of CD46 mRNA to that of the CD46 protein. Parallel immunohistochemical analysis of the limited available formalin-fixed, paraffin-embedded material indicated a positive association between CD46 at the mRNA and protein level ($p = 0.031$), with CD46 immunostaining localizing to the tumor epithelial cell population notably including prominent cell surface expression. The contrasting data between both studies might feasibly be attributed to subtle differences in primary antibodies, sample fixation or antigen retrieval techniques. A more comprehensive study investigating the expression of CD46 at the level of mRNA and protein for a number of paired graded breast cancer tissue samples is currently being planned, which should reveal the true significance of the expression of CD46 in breast tumors. Interestingly, a recent report has described reduced expression of CD46 in renal tumors where CD46 expression positively correlated with the degree of differentiation.[34] These poorly differentiated tumors expressed low levels of CD46 and contained deposits of the C activation product C3d.

Although the physiologic relevance of the statistical correlation of CD46 with IGF-IR ($p = 0.016$) is unclear, it has been reported that IGFR expression positively correlates with ER content and has been found to be a good prognostic indicator.[35] Taken collectively, our data suggest that the expression of CD46 mRNA is itself a favorable prognostic indicator in breast cancer and correlates with other such indicators.

Even though CD59 mRNA levels did not correlate significantly with other markers, expression of CD59 did correlate with CD46 (Spearmans analysis; $p = 0.01$, $r = 0.36$), perhaps reflecting the dominant protein expression patterns of these regulators in breast tumor tissue when compared to the low level of expression of

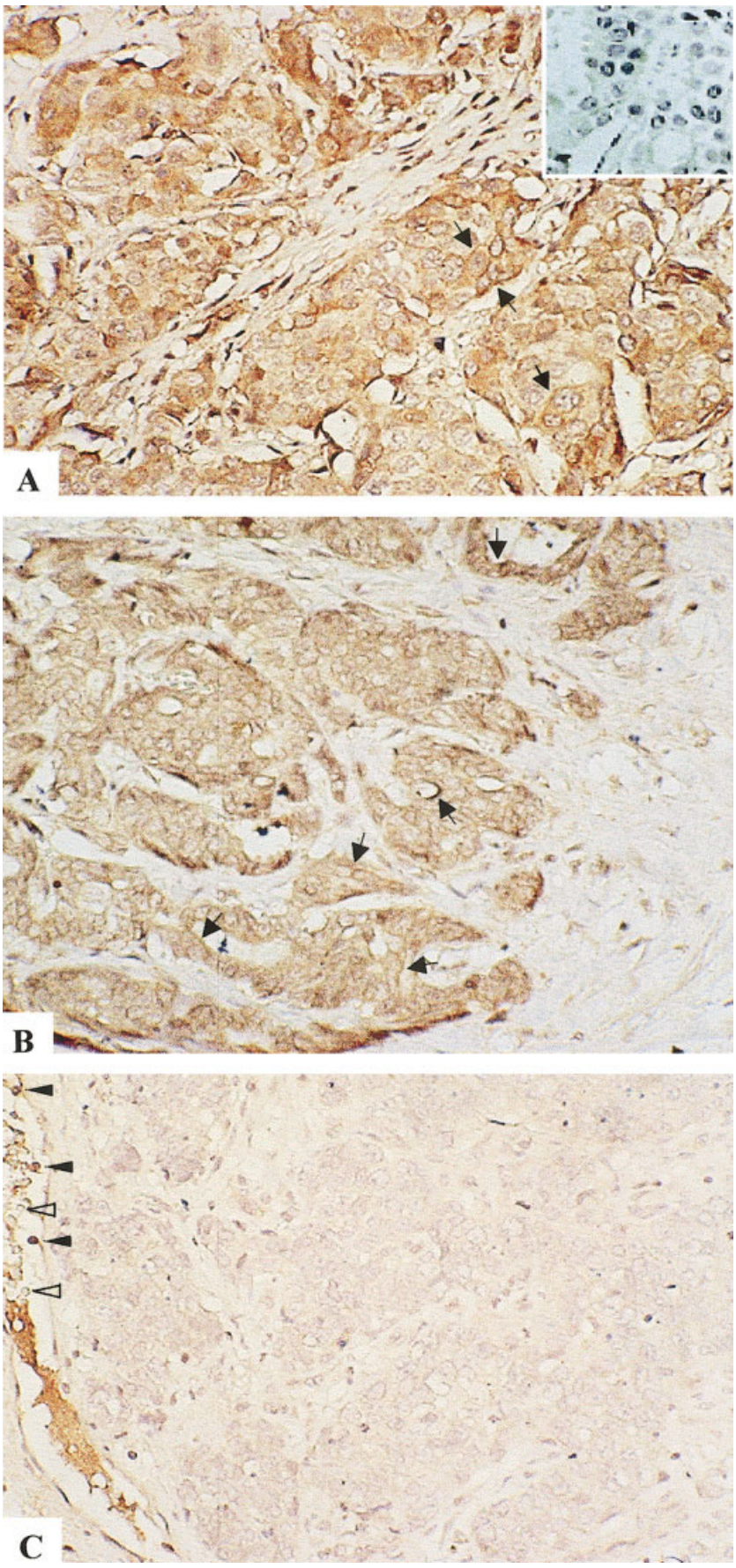

FIGURE 3 – Paraffin sections of formalin-fixed human breast tumors immunohistochemically stained for CD46 protein expression using a rabbit anti-CD46 polyclonal antibody and immunoperoxidase detection. Intense (*a*) and intermediate (*b*) tumor staining patterns for patients exhibiting relative values of 22.7 and 10.7, respectively, for CD46 mRNA expression. Prominent tumor epithelial surface membrane staining is indicated (arrow). Extremely weak CD46 tumor immunostaining (*c*) for a patient displaying a relative mRNA expression value of 2.0. Internal positive (leukocytes; closed arrowhead) and negative (erythrocytes; open arrowhead) CD46 staining are indicated. Negativity of control sections stained using an irrelevant polyclonal antibody is also shown (*a* insert). Original magnification ×25.

CD55 found by ourselves and others.[8,25] The low level of CD55 expression in breast tumors combined with the reduced expression of CD46 in ER-negative breast disease would tend to suggest that the preferred tumor treatment target for the inhibition of C regulator function might be the CD59 protein. Nevertheless, functional inhibition of the omnipresent CD46 protein might also be considered to have clinically beneficial therapeutic potential in breast cancer.

The regulation of the C system by tumor cells may be an important defense mechanism that provides an inherent advantage in the process of tissue carcinogenesis. Our data indicate that poorly differentiated breast tumors express low levels of CD55 and CD46, rendering the cells potentially susceptible to C damage. Targeting with C activating agents might therefore be an effective therapeutic strategy, particularly if this could be combined with blockade of either CD59 and/or CD46 on the tumor. We and others have shown that administration of CRP-blocking bispecific antibodies to neoplastic Raji cells[36] and renal tumor cells[19] resulted in increased C-mediated cell killing. The viability of this strategy is further strengthened by the documentation that neutralization of CRP by functional blocking antibodies significantly enhanced C-dependent cytotoxicity of cells bearing the recombinant humanized rituximab[37,38] or herceptin[10] therapeutic monoclonal antibodies.

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