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These investigations were undertaken to evaluate the utility of TA90-IC, an immune complex of 90-kD tumor-associated glycoprotein antigen and IgG antibody, compared with mammography as a screening tool for early breast cancer. Serum was drawn preoperatively from patients, coded, and tested for TA90-IC. An optical density (OD) $\geq$ 0.41 at 405 nm was considered positive based on three standard deviations above the mean of 250 normal controls. Of the 138 patients studied, 109 had an abnormal mammogram, and 29 had a normal mammogram. There was a significant difference in TA90-IC values between the 82 patients with benign lesions and the 42 patients with invasive carcinoma (0.253±0.236 and 0.436±0.209 OD, respectively, p=0.0001). In the abnormal mammogram group, 14 had ductal carcinoma in situ (DCIS) and 36 had an invasive carcinoma; among the subgroup of 41 patients with a positive TA90-IC, 31 had invasive carcinoma and 4 had DCIS. Of the 29 patients with a normal mammogram, 6 had invasive carcinoma that in 4 cases was accompanied by a positive TA90-IC. Of the 97 patients with a negative TA90-IC, 21 (22%) had a malignancy: 10 had DCIS and 11 had an invasive carcinoma. Therefore, serum TA90-IC assay appears to be a useful adjunct to mammography.

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Rishab K. Gupta 12-16-98

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INTRODUCTION

Nationwide, breast cancer has been ranked first among the five most common cancer sites for women (breast < colon < lung < ovary < cervix). Mammographic screening combined with clinical and self-breast examination are considered to be the only acceptable means for early detection of this disease. Mammography has been shown to benefit women over the age of 49. It can detect cancers at an earlier stage, resulting in an increased survival for women in this age group (1, 2). However, not all breast cancers are detected by mammography, and a false-negative rate of 16.5% has been reported (3). Also, mammography cannot distinguish benign disease from invasive disease. Therefore, many women undergo unnecessary biopsies. CA15-3, carcinoembryonic antigen (CEA), and numerous other tumor markers have been developed to aid in the diagnosis, prognosis, and early detection of breast cancer recurrence (4-18), but none has yet proved effective as an adjuvant to mammography in breast cancer screening.

During the previous years we have described a 90-kD glycoprotein tumor-associated antigen (TA90) associated with breast cancer and other solid tumors (19-23). This antigen is immunogenic in the patients. As a result TA90 is present in the serum as an immune complex (IC) with anti-TA90 IgG antibody, and we developed an enzyme-linked immunosorbent assay (ELISA) to measure TA90-IC in cancer patients (24). Since TA90 appears to be one of the aberrantly altered and/or over-expressed molecule, it certainly has a potential of being utilized as a marker for early detection and prognosis of breast cancer. In the last year's progress report, we elaborated on characterization of the TA90 (a 90kD glycoprotein tumor antigen).

Also, in our earlier study, we retrospectively examined the incidence of TA90-IC in the serum of 106 patients with known breast cancer (22). TA90-IC was identified in 63% of serum samples, compared with only 3% of healthy controls. CEA and CA15-3 were also analyzed in a subgroup of 68 serum samples: TA90-IC was identified in 55 (81%), CEA in 16 (24%), and CA15-3 in 23 (34%). Thus TA90-IC was significantly more sensitive than any of the standard tumor markers in patients with known breast cancer.

During the past year of the grant we have devoted considerable efforts to conduct investigations in accordance with the approved Statement of Work (SOW). We prospectively investigated the incidence of TA90-IC in the preoperative sera of patients with a palpable breast mass, mammographic abnormality, or bloody nipple discharge that warranted an open biopsy. TA90-IC values were then correlated with mammographic findings, standard tumor markers, and the pathology found on breast biopsy.

BODY

EXPERIMENTAL METHODS:

PATIENT POPULATION:

After obtaining informed consent, serum samples for TA90-IC ELISA were procured preoperatively in a blinded fashion from 138 women scheduled to undergo open biopsy for an abnormal mammogram, breast mass, or bloody nipple discharge. All biopsy procedures were

performed by the surgical staff of the John Wayne Cancer Institute. Biopsy specimens were examined for tumor size, tumor grade, DNA ploidy, S-phase, estrogen and progesterone receptors, and HER2neu expression. Serum samples were tested for TA90-IC in a blinded fashion (without knowledge of pathology findings). Pathology findings were then correlated with TA90-IC values in the Statistical Coordinating Unit (SCU).

TA90-IC DETECTION ASSAY:

Serum levels of TA90-IC were determined with TA90-IC ELISA, an antigen-specific assay that utilizes a murine monoclonal antibody AD1-40F4 against TA90. The monoclonal antibody and the glycoprotein antigen were prepared as previously described (19). Briefly, 100 µl of the AD1-40F4 ascites was diluted in carbonate buffer to a protein concentration of 100 µg/ml and then dispensed into wells of gluteraldehyde-activated microtiter plates (Dynex Technologies, Inc., Chantilly, VA). The plates were incubated at 4°C for 16 h and then washed with 0.025 M phosphate-buffered saline (PBS) supplemented with 0.5% Triton X-100 (PBS-TX). The plates were then blocked with 100 µl of 1% bovine serum albumin (BSA) in PBS-TX at 23°C for 1.0 hour. Serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum and 0.01 M ethylene diamine tetraacetic acid (EDTA). One hundred microliters of the diluted sample was placed in duplicate wells of the activated plates; plates were incubated at 37°C for 45 min and then washed with PBS-TX. One hundred microliters of alkaline phosphatase conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution per well was added to each test well and control well of the plates; plates were then incubated at 37°C for 45 min and washed with PBS-TX. Two hundred microliters of p-nitrophenyl phosphate (1.0 mg/ml) in 10% diethanolamine buffer as substrate was added, and the plates were incubated in the dark at 23°C for 1.0 hour. The absorbance was read at 405 nm. Each sample was tested twice with positive and negative controls. Each sample was blanked individually in the same microtiter plate. Each test plate also included controls for nonspecific protein binding and binding of conjugate to the immobilized murine monoclonal (capturing) antibody. The net optical densities of the control samples were used to generate a correction factor to normalize the net optical density of the test samples analyzed on that particular test plate. If the correction factor for a test plate fell outside the range of 0.8 to 1.2, the assay was considered invalid. The upper limit of normal for TA90-IC was 0.41 (mean ± 3 SD ELISA values of over 250 normal sera determined from previous studies). Sera with a value $\geq$0.41 OD were considered positive for TA90-IC.

STATISTICAL ANALYSIS:

The Kruskal-Wallis test was used to examine the difference between mean TA90-IC values of sera from patients with invasive carcinomas and benign lesions. The Spearman correlation coefficient was used to analyze the relationship between TA90-IC values and tumor size, tumor grade, DNA ploidy, and S-phase. The Kappa test was used to examine the consistency of TA90-IC status and estrogen receptor status, progesterone receptor status, and HER2neu expression. Pearson chi-square test was used to investigate the relationship between TA90-IC status and tumor size ($<$ 1 cm versus $\geq$ 1 cm).

RESULTS:

Biopsy specimens were characterized as benign, ductal carcinoma in situ (DCIS), or invasive carcinoma. The distribution of positive and negative TA90-IC values for each pathology is shown in Table 1. Mean TA90-IC was 0.254 ± 0.239 OD in the 82 patients with benign lesions, 0.315 ± 0.230 OD in the 14 patients with DCIS, and 0.436 ± 0.209 OD in the 42 patients with invasive lesions; the difference between benign and invasive groups was highly significant (p=0.0001).

Of the 138 patients studied, 42 had invasive carcinoma, 31 (74%) with a positive TA90-IC; 14 patients had DCIS, 4 (29%) with a positive TA90-IC; and 82 had benign lesions, 6 (7%) with a positive TA90-IC.

Of the 82 patients with benign pathology report, 72% had abnormal mammogram and 7% were positive for TA90-IC. Of the 14 patients with DCIS pathology report, all (14/14) had abnormal mammogram and 29% were positive for TA90-IC. On the contrary, of the 42 patients whose pathology reports revealed invasive carcinoma of the breast, 86% had abnormal mammogram and 74% were positive for TA90-IC.

Twenty-six patients had a normal mammogram in the face of a palpable mass, and one had a normal mammogram and a bloody nipple discharge. Of these 27 patients, none had DCIS; 6 (22%) had invasive carcinoma, 4 with a positive TA90-IC; and 21 (88%) had benign lesions, none with a positive TA90-IC. Mammography was positive in 9 patients with invasive carcinoma and negative in six patients with invasive carcinoma. Mammography was positive in 14 patients with benign lesions and negative in 21 patients with benign lesions (Table 2).

Among the 42 patients with invasive breast cancer, 27 (64%) had a positive mammogram plus a positive TA90-IC, 4 (10%) had a positive TA90-IC only, 9 (21%) had a positive mammogram only, and 2 (5%) had neither a positive mammogram nor a positive TA90-IC.

Of the 56 patients with invasive carcinoma or DCIS, 52 had lesions evaluable by size. Of the 15 patients with tumors <1 cm, 6 (40%) had a positive TA90-IC. Of the 37 patients with tumors ≥ 1 cm, 28 (82%) had a positive TA90-IC (p=0.014). Thirty-one of the 56 tumors were tested for S-phase. Of the 20 patients with a positive TA90-IC, 8 (40%) had tumors with an S-phase greater than 5%; of the 11 patients with a negative TA90-IC, only 3 (27%) had tumors with an S-phase greater than 5%. However, statistical analysis using S-phase and TA90-IC as continuous variables revealed a positive correlation between TA90-IC and S-phase (Spearman correlation coefficient =0.4011; p=0.0253) (Figure 1). There was no demonstrable association between TA90-IC value and tumor grade among 45 patients with invasive or noninvasive tumors (Spearman correlation coefficient = 0.2789, p=0.0635) or among the subgroup of 34 patients with invasive tumors (Spearman correlation coefficient=0.3040, p=0.0804).

Of the 38 tumors tested for estrogen receptors (ER) and progesterone receptors (PR), 7 were ER-negative: 6 (86%) of these were TA90-IC positive, compared with 21 (68%) of the 31 ER-positive tumors (p=0.8282). Of the 7 PR-negative tumors, 5 (71%) were TA90-IC positive, compared with 22 (70%) of the 31 PR-positive tumors (p=0.5097). Of the 34 tumors tested for

HER2/*neu* expression, 8 were positive: 6 (75%) of these were TA90-IC positive, compared with 17 (65%) of the 26 HER2/*neu*-negative tumors (p=0.3056).

The serum of 36 patients with a malignancy was tested for CEA and/or CA15-3 in addition to TA90-IC. CA15-3 was positive in 3 patients (one invasive carcinoma, 2 DCIS) of 32 patients (26 invasive carcinoma, 6 DCIS). CEA was positive in 2 patients (invasive carcinoma) of 33 patients (26 invasive carcinoma, 7 DCIS). Of the 36 patients, 24 (67%) were positive for TA90-IC, whereas only 3 (8%) were positive for CEA or CA15-3 (Table 3). One patient with DCIS had a positive CA15-3 but a negative CEA and TA90-IC. Of the 22 patients with a positive TA90-IC and invasive carcinoma, only 2 were positive for CEA or CA15-3. This confirms our earlier study demonstrating the superior sensitivity of the TA90-IC assay (22).

Of the 42 invasive carcinomas, 17 were American Joint Committee on Cancer (AJCC) Stage I, 18 were AJCC stage IIa, 5 were AJCC stage IIb, and 2 were AJCC stage IIIa. TA90-IC values were positive in 11 (65%) stage I tumors, 14 (78%) stage IIa tumors, 5 (100%) stage IIb tumors, and 1 (50%) stage IIIa tumor (Figure 2).

DISCUSSION:

Investigations undertaken during the past year have evaluated the utility of TA90-IC for identifying patients with early breast cancer in a prospective manner. Results indicate that TA90-IC shows promise as an adjunct to mammographic screening. Eighty-five percent of patients with a positive TA90-IC had a breast malignancy. Of the 97 patients with a negative TA90-IC, 76 (78%) had benign disease and 21 (22%) had a malignancy. Only 6 of 82 (7%) patients with a benign lesion had a positive TA90-IC, whereas 4 of 6 patients with invasive carcinoma and a normal mammogram had a positive TA90-IC (Figure 3). Of 29 patients with a normal mammogram, 6 (21%) had a malignancy; similarly, 21 of 97 (22%) TA90-IC values <0.41 OD were falsely negative. Five (45%) of the 11 patients with a positive mammogram had a malignancy. When TA90-IC and/or mammographic results were combined, 54 of 56 (96%) breast neoplasms were detected.

Although mammography has reduced the mortality of breast cancer by about one third in patients 50 to 69 years old, it has limitations. Not all breast cancers are demonstrable on mammography, in which case the mammogram would be falsely negative. In our study, 4 of 42 (10%) patients with invasive carcinoma had a negative mammogram. Coveney et al (3) found a 16.5% false-negative rate for mammography in 291 patients of all ages with palpable cancers. Retrospective review of false-negative mammograms showed that 30% were normal (true negatives), 20% were obvious oversights, and 50% had radiographic abnormalities that were indeterminate. A serum marker indicating whether or not a lesion was malignant should decrease the rate of false-negative mammography. This would be particularly useful in 40-49 year old patients without a palpable mass (25).

False positives are also a problem with mammography. Only 17% to 32% of non-palpable lesions found on mammography are malignant (3, 26-34). Therefore, a large percentage of open biopsies reveal benign disease. Because women younger than 50 years have a much higher rate of false-positive mammograms (27, 35), the cost of screening mammography is five times higher in the 40-49 year age group than the 50-69 year group (36). The current consensus

of the National Institutes of Health is that mammography can not universally be recommended in women between 40 and 49 years old (37). A serum marker that could distinguish between benign and malignant lesions on a mammogram should make mammography in this age group more cost-effective and beneficial.

Serum tumor markers have been described for the early detection and monitoring of metastases from a primary breast cancer (4, 5). However, many of these markers are questionable, with the possible exception of CA15-3, in regards to both diagnosis of systemic disease and response to therapy (6, 7). Tomlinson et al (13) reported 70% sensitivity, 96% specificity, and an 87% predictive value for CA15-3 in metastatic disease, but this marker does not appear to be useful in screening. Our study confirmed this finding – only 3 of 32 patients with a malignancy had a positive CA15-3. Serum markers such as TPS (14), alpha-1-antiprotease (15), YKL-40 (16), MCA (17), and c-*erb*B-2 (8, 10-12) have shown promise in predicting survival and monitoring disease regression or recurrence, but are not suitable for screening.

CONCLUSION

Results of these investigations indicate that TA90-IC is a potentially useful screening marker in patients with early breast carcinoma. Although a higher proportion of stage IIa patients were TA90 positive, 11 of 17 stage I patients were positive for TA90-IC. This means that TA90-IC is detectable in the blood of patients with smaller, premetastatic tumors. Although this marker is not 100% sensitive or specific, it complements mammography by identifying cancers by an independent means. In our study, we showed that TA90-IC can identify 67% of tumors not visualized on a mammogram. Of course, these tumors were palpable and did not present a diagnostic dilemma. In this group of patients, all of whom had an indication of breast biopsy, TA90-IC proved to be a useful adjunct to mammography. The role of TA90-IC in breast cancer screening will be determined by prospectively examining whether a positive serum TA90-IC value can be used with mammography for routine screening of women at risk of breast cancer.

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Table 1. *TA90-IC Results by Pathology*

	BENIGN	*DCIS*	*INVASIVE*	*TOTAL*
TA90-IC <0.41	76 (93%)	10 (71%)	11 (26%)	97 (70%)
TA90-IC ≥0.41	6 (7%)	4 (29%)	31 (74%)	41 (30%)
TOTAL	82	14	42	138

Table 2. *TA90-IC and mammographic results according to pathology of the breast lesion*

	Pathology of the Breast Lesion		
Screening test results	*Benign*	*DCIS*	*Invasive cancer*
TA90-IC (+) Mammography+	6	4	27
TA90-IC (+) Mammography-	0	0	4
TA90-IC (-) Mammography+	53	10	9
TA90-IC (-) Mammography-	23	0	2

Table 3. *Incidence of three serum markers (TA90-IC, CEA, CA15-3) in sera of patients with DCIS or invasive breast cancer.*

	Histopathology of breast lesion	
Marker status	*DCIS*	*Invasive cancer*
TA90-IC (+) CEA/CA15-3+	0	2
TA90-IC (+) CEA/CA15-3-	2	20
TA90-TC (-) CEA/CA15-3+	1	0
TA90-IC (-) CEA/CA15-3-	4	7

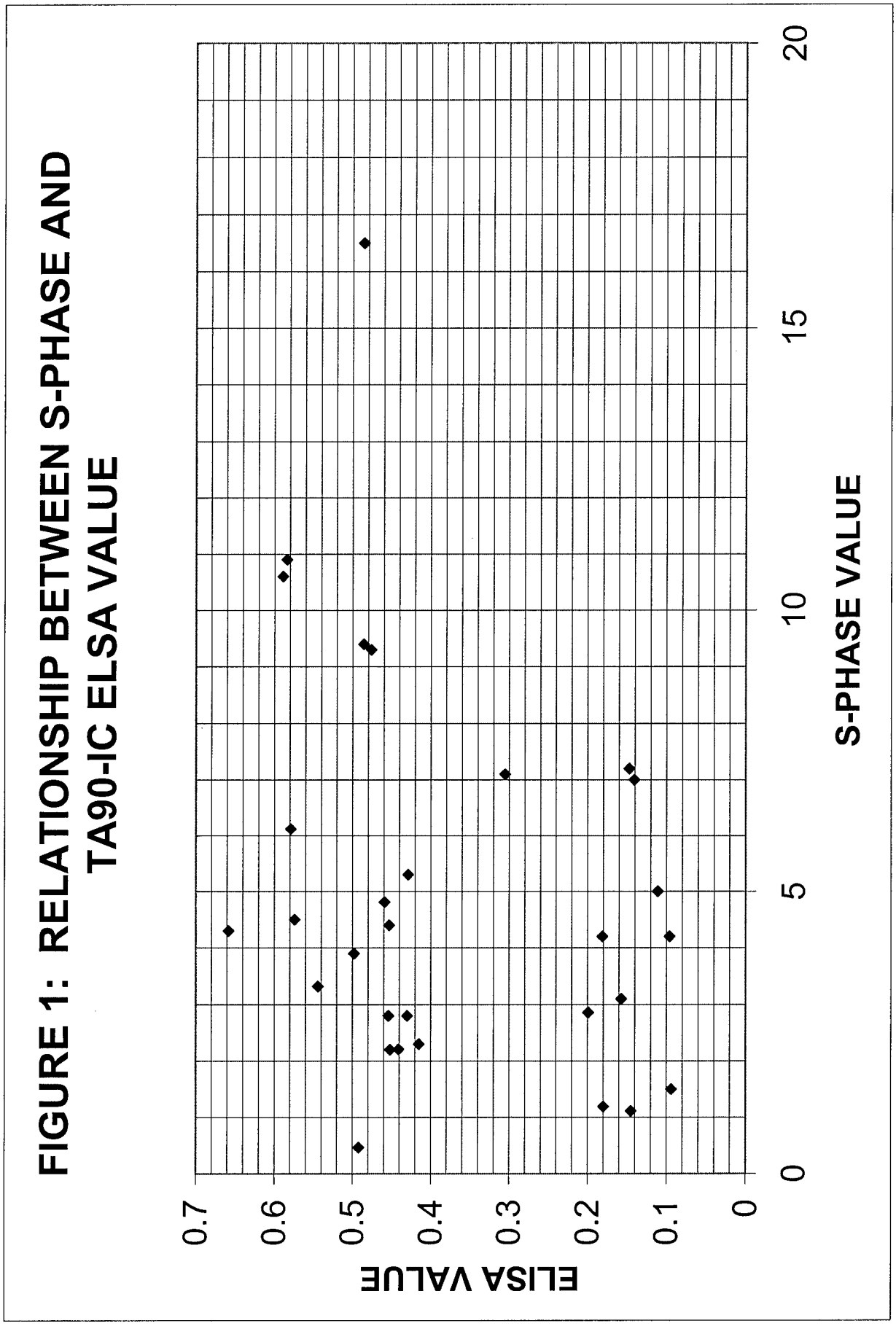


FIGURE 1: RELATIONSHIP BETWEEN S-PHASE AND TA90-IC ELSA VALUE
ELISA VALUE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
S-PHASE VALUE

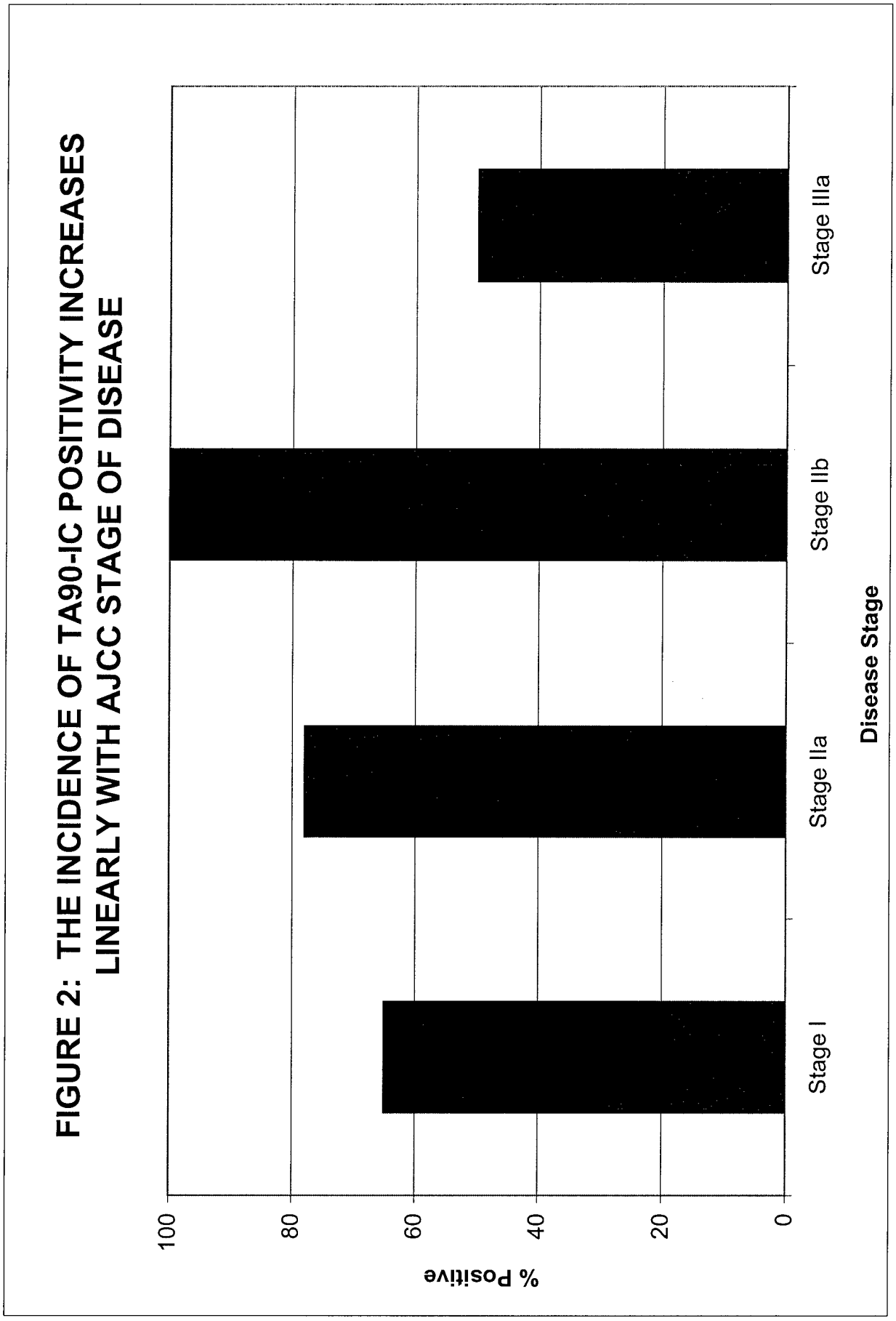
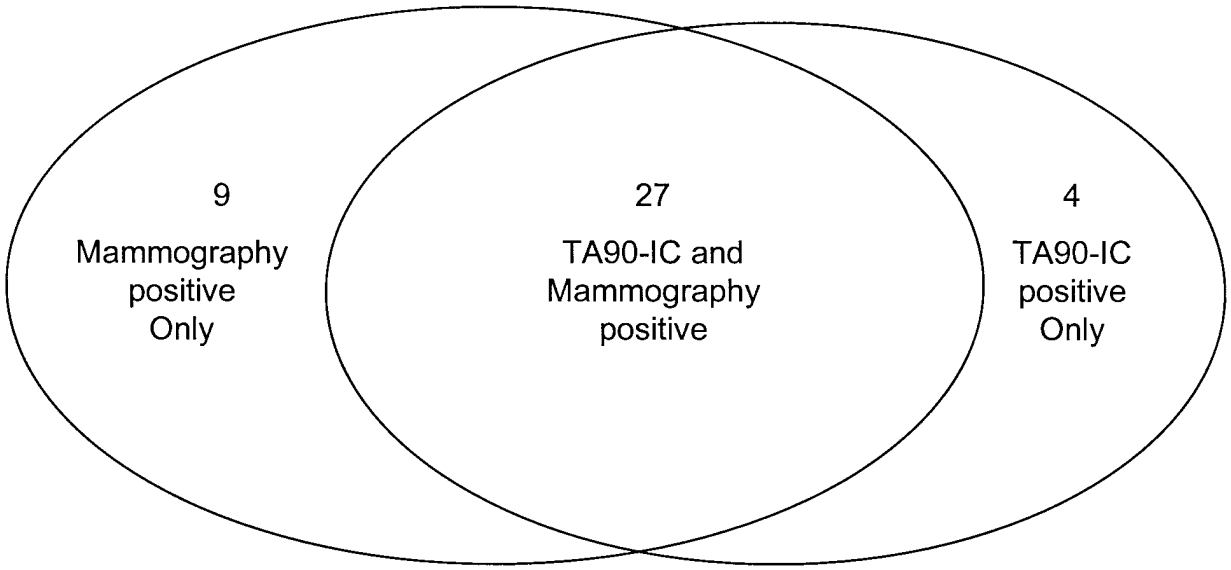


FIGURE 2: THE INCIDENCE OF TA90-IC POSITIVITY INCREASES LINEARLY WITH AJCC STAGE OF DISEASE
% Positive
100
80
60
40
20
0
Stage I
Stage IIa
Stage IIb
Stage IIIa
Disease Stage

FIGURE 3: OVERLAP BETWEEN RESULTS OF MAMMOGRAPHY AND TA90-IC ELISA IN 40 PATIENTS WITH INVASIVE BREAST CARCINOMA. TWO ADDITIONAL PATIENTS WITH INVASIVE CARCINOMA HAD NEGATIVE MAMMOGRAPHIC FINDINGS AND A NEGATIVE TA90-IC ELISA.

Invasive carcinoma: N=42



2 patients: mammogram and TA90-IC negative