

Urinary 2/16 α -hydroxyestrone Ratio: Correlation with Serum Insulin-like Growth Factor Binding Protein-3 and a Potential Biomarker of Breast Cancer Risk[§]

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Abstract

Metabolism of estradiol occurs via two mutually exclusive hydroxylative pathways, yielding metabolites of divergent biological properties. 2-hydroxyestrone (2OHE1) is anti-estrogenic while 16 α -hydroxyestrone (16 α OHE1) is a potent estrogen. The ratio of 2OHE1 to 16 α OHE1 (2/16 α -OHE1 ratio) represents the net in vivo estrogenic activity. In this study, we sought to determine if the urinary 2/16 α -OHE1 ratio could be a predictor of breast cancer risk and the factors which influence this ratio. Variables analysed included age at diagnosis, menopausal status, parity, use of oral contraceptives, body mass index, serum levels of insulin-like growth factor-I (IGF-I), IGF binding proteins (BPs) and the presence of breast cancer. Serum and urine were collected from 65 breast cancer patients and 36 controls after an overnight fast. Urinary estrogen metabolites were measured by enzyme immunoassays while serum levels of IGF-I, BP-1 and BP-3 were determined by immunoradiometric assays. 2OHE1 levels and 2/16 α -OHE1 ratios were significantly lower ($P < 0.05$) while 16 α OHE1 levels were higher ($P < 0.01$) in cancer patients. Multiple linear regression analysis showed that levels of urinary metabolites were influenced by parity and breast carcinoma. 2/16 α -OHE1 ratio correlated positively with serum BP-3 level ($P = 0.03$). By multiple logistic regression, 2/16 α -OHE1 ratio was the most significant factor predictive of breast cancer. The odds ratio for women with higher 2/16 α -OHE1 ratios was 0.10 (0.03 - 0.38, 95% confidence interval). In conclusion, the profile of urinary estradiol metabolites was distinctly altered in breast cancer patients. In addition, BP-3 may be a potential mechanism by which estradiol metabolites influence breast cancer progression. As 16 α OHE1 has been shown to initiate neoplastic transformation of mammary epithelial cells, the 2/16 α -OHE1 ratio may serve as a biomarker of increased risk of breast cancer.

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Introduction

Breast cancer is the most common cancer among women in Singapore with age-standardised incidence rates increasing markedly from 26.8 to 38.7 per 100 000 per year over the last decade.* While many recognised risk factors exist for the development of breast cancer such as demographic factors (personal and family history of breast cancer[>] and endocrine factors (age of menarche, at first pregnancy and at menopause), these factors are present in only a small proportion of breast cancer patients. There is a need for a more sensitive and specific biomarker of breast cancer risk.

Estradiol has long been thought to play an important role in the development and progression of breast cancer. However, the precise mechanism by which estrogen promotes breast cancer development remains controversial despite extensive human and animal experimental research. Metabolism of estradiol is primarily oxidative via 2 mutually exclusive hydroxylative pathways with hydroxylation at the C2 or 16 α position. The products of these 2 pathways have divergent biological properties. The main 2-hydroxyestrogen, 2-hydroxyestrone (2OHE1), has no peripheral estrogenic activity but plays a regulatory role in neuroendocrine mediated

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events.² In contrast, the principal 16 α -hydroxyestrone, 16 α -hydroxyestrone (16 α OHE1), demonstrates strong estrogenic activity comparable to the parent estradiol.^{2,3} Thus, the ratio of 2OHE1 to 16 α OHE1 (2/16 α -OHE1 ratio) reflects the net *in vivo* estrogenic stimulus.⁴ In addition, recent *in vitro* studies have shown that 2OHE1 acts as an estrogen antagonist and suppresses proliferation of breast cancer cells^{5,6} whereas 16 α OHE1 induced genotoxic responses. Furthermore, it has been shown that 16 α OHE1 levels are increased in breast cancer patients^{7,8} and in women at high risk for breast cancer.⁹ Therefore, a decreased 2/16 α -OHE1 ratio may represent a propensity towards uncontrolled proliferation of breast epithelial cells.¹⁰

Insulin-like growth factor-I (IGF-I) is also implicated in the pathogenesis of breast cancer. IGF-I is a potent mitogen *in vitro*^{11,12} and IGF-I receptors are present on the cell surface of various breast cancer cell lines.¹³ *In vivo*, most of circulatory IGF-I are bound to its binding proteins (BPs) which modulate IGF activity at the tissue level. Previous reports have suggested possible associations between plasma estradiol and the IGF system. Plasma estradiol levels have been reported to correlate with plasma IGF-I levels in premenopausal women¹⁴ while studies evaluating the influence of exogenous estrogens on plasma IGF-I have been inconsistent.¹⁵⁻¹⁷ *In vitro* investigations suggest that IGF-I stimulates peripheral aromatase activity,¹⁸ the pivotal enzyme for estrogen synthesis in postmenopausal women.¹⁹ However, a recent small study found no correlation between the urinary estrogen metabolites and plasma IGF-I or BP-1 levels.²⁰ No study has examined for a possible relationship between BP-3, the predominant binding protein for IGF-I, and the estradiol metabolites.

The objectives of this study were to determine if the urinary estrogen profile was indeed different in patients with breast cancer and if the 2/16 α -OHE1 ratio could be a potential predictor of breast cancer. We also attempted to determine the factors which could influence the urinary estrogen profile.

Materials and Methods

Patients

One hundred and one patients were enrolled into this study, comprising 65 breast cancer patients and 36 controls. Insulin-dependent diabetics, patients with hepatic dysfunction or loss of more than 5% of pre-morbid weight were excluded. Cases were women with histologically proven primary breast cancer treated in our institution from January 1995 to May 1996. None of these patients received preoperative chemotherapy or radiotherapy. Controls were randomly selected from women who underwent breast biopsies over the same period for mammographic abnormalities detected in a national breast screening

project. All patients were confirmed to have benign breast disease on histology.

Informed consent was obtained from all patients. All women completed a questionnaire by interview and information on the following was sought: age at diagnosis, parity, menopausal status and use of oral contraceptives. Patients were considered postmenopausal if spontaneous cessation of menstruation had occurred for more than six months or after bilateral oophorectomy. Measurements of weight and height were taken by the same trained nurse using verified scales and calibrated tapes. Body mass index (BMI, Quetelet's Index) was calculated as weight divided by height squared (kg/m²) for nutritional assessment.

Blood and Urine Sampling

Blood and urine samples were collected after an overnight fast without any dextrose infusion prior to surgery. Serum was separated by centrifugation within 30 minutes and stored at -70°C until analysis. 30 mg of ascorbic acid was added to each 20 ml urine sample to prevent oxidation of the estrogen metabolites, placed immediately on ice (4°C) and stored at -20°C within 8 hours until analysis. For premenopausal patients, samples were collected randomly during the menstrual cycle.

Assays

Urine levels of 2OHE1 and 16 α OHE1 were determined by a competitive, solid-phase enzyme immunoassay (EIA, ESTRAMET™ 2/16, Immuna Care Corporation, Bethlehem, USA) after deconjugation of the samples as previously described.⁴ The sensitivity of 16 α OHE1 assay was 0.05 ng/ml while that of the 2OHE1 was 0.15 ng/ml (after dilution of 1:40 for assay). The intra- and inter-assay coefficients of variation (CV) for the metabolite ratio were 4.0% and 7.0%, respectively.

Serum concentrations of IGF-I, BP-1 and BP-3 were measured by immunoradiometric assays (IRMA, Diagnostic Systems Laboratories, Inc., Webster, TX, USA) in 79 of the patients (53 cases and 26 controls). The IRMA is a non-competitive assay in which the analyte is sandwiched between two antibodies and the second antibody is radiolabelled for detection. IGF-I levels were determined after acid-ethanol extraction of 50 μ l of serum as previously described.²¹ The minimal detection limit was 0.80 ng/ml. The intra- and inter-assay CV were 3.0% and 1.5%, respectively. BP-1 levels were measured using 25 μ l of serum with a minimum detection limit of 0.33 ng/ml. The intra- and inter-assay CV were 4.6% and 6.0%, respectively. 10 μ l of serum was diluted 100-fold before determination of BP-3 levels. The sensitivity of this assay was 0.5 ng/ml, and the intra- and inter-assay CV were 3.2% and 0.5%, respectively.

Statistical Analysis

Urinary levels of 2OHE1 and 16 α OHE1, 2/16 α -OHE1 ratios, and serum levels of IGF-1, BP-1 and BP-3 of cancer cases and controls were compared using Student's two-tailed t-test. Values were expressed as mean $\pm$ standard error of mean (SEM). Forward conditional logistic regression was used to find a suitable model to predict breast cancer and to adjust for possible co-linearity of independent variables. The criterion for entry of the variable into the model was set at a statistic score of 0.05. The final model was adjusted for the influence of confounding factors due to age, parity (nulliparous versus parous), menopausal status, use of oral contraceptives (never versus ever) and BMI. Stepwise multiple linear regression was used to test for correlation between the urinary variables and the other factors. *P* values less than 0.05 were considered statistically significant. Results were analysed using the SPSS package.

Results

The characteristics of the patients are shown in Table I. There was no significant difference in characteristics between the cases and controls.

The pattern of urinary excretion of the estrogen metabolites was significantly different between the two groups (Table II). Controls excreted a higher level of 2OHE1 while the mean level of the 16 α OHE1 was greater in cancer patients. The 2/16 α -OHE1 ratio was significantly lower in the cancer group (*P* = 0.000). As pre- and postmenopausal women have differing ovarian function and plasma estradiol levels vary during the menstrual cycle in premenopausal women, a subset analysis considering only postmenopausal patients was performed. The results showed similar significant differences between the two groups (Table II).

Serum IGF-1 and IGFBP-1 levels were not significantly different between cases and controls (Table II). In contrast, BP-3 levels were significantly lower in patients with breast cancer compared to controls (*P* = 0.001).

Risk of Breast Cancer

For risk of breast cancer, only urinary 2/16 α -OHE1 ratio (*P* < 0.001) and serum BP-3 (*P* = 0.006) were selected in the logistic regression model. Decreases in both variables were observed in cancer patients. The adjusted odds ratio for breast cancer are shown in Table III.

Correlation between Urinary Estrogen Metabolites and Other Variables

By stepwise multiple linear regression, the 2/16 α -OHE1 ratio and urinary 2OHE1 levels were found to correlate negatively with parity and the presence of breast cancer (Table IV). Urinary 16 α OHE1 was positively correlated with breast cancer alone. When the analysis was repeated with the exclusion of the presence

TABLE I: CHARACTERISTICS OF ALL PATIENTS AND TUMOURS

All patients (n = 101)	Cases (n = 65)	Controls (n = 36)	
Characteristics	Number (%)	Number (%)	<i>P</i> value
Age† (years)	54.0 $\pm$ 1.2	54.8 $\pm$ 0.9	0.57*
Body weight† (kg)	59.6 $\pm$ 2.1	56.5 $\pm$ 0.5	0.30*
BMI† (kg/m ²)	24.4 $\pm$ 0.6	23.3 $\pm$ 0.5	0.22*
Menopausal status			1.00**
Pre	23 (35.4)	12 (33.3)	
Post	42 (64.6)	24 (66.7)	
Parity			1.00**
Nulliparous	9 (13.8)	5 (13.9)	
Parous	56 (86.2)	31 (86.1)	
Use of Oral Contraceptives			0.68**
Never	49 (75.4)	25 (69.4)	
Ever	16 (24.6)	11 (30.6)	
Cancers Only (n = 65)			
Stage (TNM Staging System)			
Tis	6 (9.2)		
I	15 (23.1)		
II	38 (58.5)		
III	5 (7.7)		
IV	1 (1.5)		
Estrogen Receptor‡			
Negative	28 (43.1)		
Positive	30 (46.2)		
Progesterone Receptor‡			
Negative	32 (49.2)		
Positive	26 (40.0)		
Grade§			
Low	7 (10.8)		
Moderate	26 (40.0)		
High	22 (33.8)		

* *P* values by two-tailed unpaired *t*-test

** *P* values by chi-square or Fisher's exact test

† Values are expressed as mean $\pm$ standard error of mean

‡ Hormonal receptor status was not assessed in 1 invasive tumour and 6 cases of ductal carcinoma-in-situ

§ 4 invasive tumours and 6 cases of ductal carcinoma-in-situ were not graded

of cancer, positive correlation was demonstrated between BP-3 level and 2/16 α -OHE1 ratio (B coefficient **4.86**, *P* = **0.032**).

Discussion

The EIA for 2OHE1 and 16 α OHE1 in human urine samples is a non-invasive and reliable method of measuring the estrogen metabolites. The kit measures the 2 metabolites simultaneously using the same sample aliquot, thereby avoiding inter-assay variation. Furthermore, by expressing the results as a ratio, the need to account for dilution factor and for standardisation using creatinine values was eliminated.¹⁰ Ideally, urine samples should be obtained from a 24-hour collection. In this

TABLE II: COMPARISON OF THE LEVELS OF URINARY ESTROGEN METABOLITES, SERUM IGF-1, IGFBP-1 AND IGFBP-3 BETWEEN CASES AND CONTROLS

Urinary Factors (ng/ml)	Cases	Controls	*P value
All Patients	(n = 65)	(n = 36)	
2-hydroxyesterone	5.4 ± 0.6	10.0 ± 2.0	0.030
16α-hydroxyesterone	8.5 ± 0.7	4.7 ± 0.5	0.000
2/16α-OHE1 ratio	0.7 ± 0.1	2.0 ± 0.3	0.000
Postmenopausal patients	(n = 42)	(n = 24)	
2-hydroxyesterone	4.5 ± 0.6	10.9 ± 2.8	0.030
16α-hydroxyesterone	7.3 ± 0.8	4.4 ± 0.4	0.001
2/16α-OHE1 ratio	0.7 ± 0.1	2.2 ± 0.5	0.005
Serum Factors (ng/ml)	(n = 53)	(n = 26)	
IGF-1	145.7 ± 12.3	166.7 ± 16.8	0.325
IGFBP-1	23.0 ± 2.8	33.8 ± 6.1	0.118
IGFBP-3	2027 ± 72	2647 ± 161	0.001

Values are expressed as mean ± standard error of mean
* P values by two-tailed unpaired t-test; P values <0.05 are in bold

TABLE IV: CORRELATION BETWEEN URINARY ESTROGEN METABOLITES, SERUM IGF-I AND BPS, DEMOGRAPHIC INDICES AND ENDOCRINE FACTORS; STEPWISE MULTIPLE LINEAR REGRESSION

	2-OHE1		16α-OHE1		2/16α-OHE1 Ratio	
	B coefficient	P value	B coefficient	P value	B coefficient	P value
Parity	-6.54	0.014	-	-	-1.50	0.000
Breast Cancer	-7.05	0.001	3.28	0.011	-1.55	0.000
Serum BP-3*	-	-	-	-	4.86	0.032

Only statistically significant results are shown. Other variables considered were age at diagnosis, menopausal status, use of oral contraceptives, body mass index and serum levels of IGF-I, BP-1 and BP-3.
* Correlation with 2/16α-OHE1 ratio was statistically significant when the presence of breast cancer was excluded from the analysis.

study, fasting spot urine samples were utilised and no attempt was made to evaluate the urinary estrogen profile at specific times of the menstrual cycle. To address the latter, a possible confounding factor, statistical analysis was repeated for the subset of postmenopausal patients only.

A host of physiological factors can influence serum levels of IGF-I, BP-1 and BP-3, such as age, menopause, nutritional status, food intake and insulin.²²⁻²⁴ To minimise the effects of confounding variables, insulin-dependent diabetics and patients with metabolic disorders from liver disease or significant loss of body weight were excluded. There was no difference in the distribution of age, menopausal status and nutritional status between the cancer and control groups. Only fasting blood samples were utilised.

The results of this study indicate that the urinary estrogen profiles between breast cancer patients and

TABLE III: INFLUENCE OF URINARY 2/16α-OHE1 RATIO AND SERUM IGFBP-3 ON RISK OF BREAST CANCER

Variables	B coefficient	P value	Adjusted odds ratio (95% CI)
2/16α-OHE1 Ratio*			
continuous variable	-2.31	0.001	0.10 (0.03 - 0.38)
≤0.9 versus >0.9 (50th percentile)	1.86	0.001	6.45 (2.05 - 20.3)
IGFBP-3* (ng/ml)			
Continuous variable	-0.001	0.006	0.999 (0.998 - 1.00)
>2094 versus ≤2094 (50th percentile)	-1.59	0.006	0.20 (0.07 - 0.63)

Forward logistic regression model considered age at diagnosis, menopausal status, parity, use of oral contraceptives, body mass index, urinary levels of 2OHE1 and 16αOHE1, 2/16α-OHE1 ratio, and serum levels of IGF-1, BP-1 and BP-3. Only results of variables considered statistically significant are shown.
* 2/16α-OHE1 ratio and BP-3 were analysed as both continuous and binary variables (at 50th percentile).

non-cancer women are significantly different. The decrease in 2OHE1 and/or increase in 16αOHE1, with resultant decrease in 2/16α-OHE1 ratio, in cancer patients are consistent with the hypothesis that specific metabolites of endogenous estrogens are determinants of the risk of breast cancer. The difference in 2/16α-OHE1 ratio between the two groups was still significant when only postmenopausal patients were considered. Other investigators have shown that formation of 16αOHE1 is specifically and significantly elevated in breast cancer patients and in women at high risk for developing breast cancer. Increased formation of 16αOHE1 has great biological significance because it is a potent estrogen agonist with unique properties. It is capable of binding covalently to the estrogen receptor, thereby prolonging its biological effects.²⁵ It also has full biological availability because of its lack of affinity for sex hormone binding globulin (SHBG).² In vitro experiments have shown that 16αOHE1 induces genotoxic responses and promotes anchorage-independent proliferation of breast cancer cells, suggesting that it could function as an initiator in pre-neoplastic transformation of mammary epithelial cells.²⁵ On the contrary, 2OHE1 has anti-estrogenic effect.^{5,6} It suppresses proliferation by decreasing unscheduled DNA synthesis and proliferation of anchorage-independent growth.²⁶

With the preceding evidence and the fact that urinary 2/16α-OHE1 ratio is a reflection of the relative metabolism of estradiol via the two mutually exclusive pathways, a decrease in the ratio may antedate clinically evident breast carcinoma. It is therefore not surprising that the 2/16α-OHE1 ratio was selected as a predictor of breast cancer after adjusting for possible confounding factors such as age, parity, menopausal status, use of oral contraceptives and BMI. In addition, in this study,

the 2OHE1 level and 2/16 α -OHE1 ratio were found to be negatively correlated while 16 α OHE1 positively correlated with the presence of breast cancer. A decreased ratio was associated with increased risk of breast cancer. Hence, the urinary 2/16 α -OHE1 ratio is a potential biomarker for the risk of developing breast carcinoma.

Interestingly, serum BP-3 level was significantly decreased in cancer patients (2027.2 ng/ml $\pm$ 72.2, mean $\pm$ SEM) and was also selected as a significant predictor of breast cancer in the logistic regression model. This is consistent with a previous study which reported that serum BP-3 level was significantly associated with risk of breast cancer, the odds ratio in women with BP-3 levels higher than 2066 ng/ml being 0.18 (0.05-0.55, 95% CI).²⁷ As BP-3 is the predominant BP for IGF-I, a decrease in BP-3 may result in an increase in the bioavailability of IGF-I. Sufficient evidence exists to support a growth inhibitory role for BP-3 on breast carcinoma. IGFBP-3 attenuated the proliferation of MCF-7 cells even in the absence of estrogens.^{28,29} Marked decrease in proliferative activity was demonstrated in cells constitutively over-expressing BP3 after gene transfection.³⁰ Decreased BP-3 production has also been associated with epidermal growth factor-stimulated proliferation.³¹ Recent evidence suggests that BP-3 can exert its anti-proliferative effects through BP-3 specific receptors, independent of its IGF-I binding properties.

Another striking finding in this study was the positive correlation between serum BP-3 levels and the urinary 2/16 α OHE1 ratios. No correlation was found between IGF-I or BP-1 and the urinary estrogens, similar to a previous report.¹⁵ However, BP-3 levels were not assessed in that study. As discussed earlier, high levels of BP-3 and 2/16 α OHE1 ratio both confer protection against breast cancer. Significant elevation of serum BP-3 levels has been demonstrated in breast cancer patients treated with the anti-estrogen Tamoxifen.³² However, this correlation may only be associative. Nonetheless, in view of the anti-proliferative effects of BP-3, this finding suggests that IGF system, in particular BP-3, may be a mechanism whereby the estradiol metabolites influence cellular proliferation of breast carcinomas.

The negative correlation between parity and both 2OHE1 and 2/16 α -OHE1 ratio was rather perplexing. Parous women were found to have lower levels of 2OHE1 and lower 2/16 α -OHE1 ratios as compared to the nulliparous. This is contradictory to epidemiological evidence that nulliparous women are at higher risk of developing breast cancer. The exact mechanism by which parity confers protection against breast cancer requires further clarification.

In conclusion, this study demonstrated that the profiles of urinary estrogen metabolites are distinctly altered in patients with breast cancer as compared to

normal controls. The 2/16 α -OHE1 ratio may serve as a biomarker of increased risk of breast cancer. In addition, IGFBP-3 may be a potential mechanism by which the estradiol metabolites influence breast cancer progression. Further prospective cohort studies are necessary to validate these findings. The urinary 2/16 α -OHE1 ratio may become a useful and simple tool for screening to identify patients at high risk of developing breast carcinoma.

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