Molecular Significance of Excess Body Weight in Postmenopausal Breast Cancer Patients, in Relation to Expression of *Insulin-like Growth Factor I Receptor* and *Insulin-like Growth Factor II* Genes

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A number of epidemiological and clinical studies have revealed that excess body weight increases the risk of postmenopausal breast cancer and also adversely affects subsequent malignant progression. To elucidate the molecular mechanisms underlying these observations, we examined mRNA expression of various genes in normal (non-cancerous) mammary gland and cancer tissue of Japanese patients with primary breast cancer, in association with their body mass index (BMI). On the basis of analysis of 106 breast cancer patients, we found that mRNA expression of insulin-like growth factor I receptor (IGF-IR) and insulin-like growth factor II (IGF-II) in the normal mammary gland showed a significant and positive association with increased BMI among postmenopausal patients. Furthermore, the positive association of increased BMI with IGF-IR mRNA expression was also found in postmenopausal breast cancer tissue, while this association was not observed among premenopausal patients. In addition, increased mRNA expression of cyclin D1 and bcl-2 was observed in association with increased mRNA levels of IGF-IR among the patients regardless of menopausal status. These findings suggest that the molecular consequence of the increased BMI is the increased expression of IGF-II and IGF-IR, resulting in development of postmenopausal breast cancer and its progression mediated through modulation of the cell cycle and apoptosis.

Key words: Breast cancer — Body mass index — Insulin-like growth factor II — Insulin-like growth factor I receptor

Numerous epidemiological studies have shown that increased body weight is a consistent risk factor for breast cancer among postmenopausal women, not only in Western countries, but also in Japan, where women tend to have a lower body mass index (BMI) than Caucasians.[1–7] However, increased body weight is not a risk factor in premenopausal breast cancer. In addition, increased body weight is also associated with poorer prognosis of breast cancer.[7–11] Thus, increased body weight in postmenopausal women is thought to influence both development of breast cancer and its progression, although most studies have so far focused on estrogen as a mediator of obesity, i.e., the increased levels of bioavailable estrogen due to activation of aromatase in adipose tissue and decrease of sex hormone-binding globulin (SHBG) in postmenopausal obese women.[7, 12, 13] However, increased BMI persisted as a significant risk factor even after adjustment for serum estrogen levels; obesity adversely affects the prognosis of postmenopausal breast cancer even independently of estrogen receptor (ER) status.[11, 13] Therefore, increased BMI

may influence the genesis of breast cancer and its malignant progression through other mechanisms than its effect on the peripheral conversion to estrogen. However, little investigation has been carried out on this point.

Insulin-like growth factors, IGF-I and IGF-II, are candidates to be involved in this mechanism, since they are potent mitogens acting on breast epithelium by autocrine and/or paracrine pathways.[14–16] The mitogenic effects of IGFs on cells mediated by insulin-like growth factor I receptor (IGF-IR), and critical roles of IGFs and IGF-IR in malignant transformation of breast epithelium and progression of breast cancer, have been documented in numerous studies.[17–20] Specifically, fibroblasts which were derived from mouse embryos lacking IGF-IR cannot be transformed by introduction of any of numerous oncogenes.[21–23]

In this experiment, we intended to clarify the molecular mechanisms through which BMI influences the malignant development of breast epithelium and the progression of cancer as a host factor. Doing so would help in the understanding of the mechanisms underlying epidemiological findings in breast cancer, and might provide biological markers useful for the evaluation of individual risk and efficacy of intervention trials aiming at breast cancer pre-

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vention. We first found that mRNA expression of IGF-IR and IGF-II in normal (non-cancerous) breast epithelium of postmenopausal breast cancer patients showed a positive and significant association with BMI, after examining mRNA expression of various genes in association with BMI. Second, the examination of breast cancer tissue revealed that mRNA expression levels of IGF-IR in high BMI patients were higher than those in low BMI patients. We further examined the association of IGF-IR and IGF-II with the expression of other genes and found that mRNA expression of cyclin D1 and bcl-2 was positively associated with that of IGF-IR, a result consistent with *in vitro* studies.[24–27] Our study indicates that increased BMI of postmenopausal women is related to increased expression levels of IGF-IR and IGF-II mRNA in breast cancer and also in normal mammary gland, which may result in modulation of the cell cycle and apoptosis.

MATERIALS AND METHODS

Tissue samples Surgical specimens of non-cancerous and breast cancer tissue were collected from patients with primary breast cancer at Saitama Cancer Center Hospital; the non-cancerous samples were the most distal "normal" mammary gland from the tumor. Samples were immediately incised in 1 ml of denaturing solution (4 *M* guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7; 0.5% sarcosyl, 0.1 *M* 2-mercaptoethanol) and stored at −80°C, followed by isolation of RNA. DNA was also extracted from the same samples. We thus established a DNA/RNA bank of breast cancer, along with the epidemiological and clinical data of patients. This bank was approved by the Institutional Review Board of Saitama Cancer Center. ER and progesterone receptor (PgR) protein assays for tumor were performed with EIA kits. Values >5 fmol/mg protein were considered positive for both receptors.

Epidemiological data Using a standardized questionnaire, breast cancer inpatients were interviewed at Saitama Cancer Center Hospital on their menstrual state and history; history of pregnancy, abortion, childbirth and lactation; history of diseases and drug use; family history of breast cancer; present and past body measurements; history of cigarette consumption, alcohol use, consumption of various types of tea, coffee and meat; and experience of physical activity. BMI at diagnosis of breast cancer was used in this study.

Isolation of RNA and semi-quantitative RT-PCR Total RNA was isolated from about 0.1 g of human mammary tissue according to the acid-guanidinium-phenol-chloroform method. RT-PCR was carried out using the TaKaRa RNA PCR kit ver. 2.1 (TaKaRa Shuzo Co., Ltd., Tokyo) as described in our previously report, in which a linear increase of radio-labeled PCR products with increasing amounts of RNA was confirmed, indicating that this semi-

quantitative RT-PCR method can discriminate relative mRNA levels of genes.[28]

Oligonucleotides used in PCR amplification were as follows: IGF-I sense, 5′-TCA ACA AGC CCA CAG GGT ATG-3′ and IGF-I antisense, 5′-TGC ACT CCC TCT ACT TGC GTT-3′; IGF-II sense, 5′-TCC TGG AGA CGT ACT GTG CT-3′ and IGF-II anitisense, 5′-CTT GGG TGG GTA GAG CAA TC-3′; IGF-IR sense, 5′-CAG TCC CAC AGT TGC TGC AA-3′ and IGF-IR antisense, 5′-CCG AAG GTC TGT GAG GAA GA-3′; glyceraldehyde phosphate dehydrogenase (GAPDH) sense, 5′-ACA TCG CTC AGA CAC CAT GG-3′ and GAPDH antisense 5′-GTA GTT CAG GTC AAT GAA GGG-3′; cyclin D1 sense, 5′-AAC TAC CTG GAC CGC TTC CT-3′ and cyclin D1 antisense 5′-CAG GTT CCA CTT GAG CTT GT-3′; and bcl-2 sense, 5′-GAC TTC GCC CAG ATG TCC AG-3′ and bcl-2 antisense 5′-TAC CTT GTG GCT CAG ATA GG-3′. The primers were designed to sandwich at least one intron for specific detection of mRNA. IGF-I mRNA is known to have two transcripts, IGF-IA and IGF-IB.[29] Although we measured IGF-IA and IGF-IB mRNA expression for several samples of breast cancer before starting this study, the expression levels of these transcripts were almost the same. We therefore used the specific primers for IGF-IA mRNA in this study. The prepared RNA (1 *μ*g) was reverse-transcribed to synthesize cDNA using random hexamers at 42°C and then subjected to PCR amplification with specific primers (0.4 *μ*g) and 3 *μ*Ci of [α-^{32}P]dCTP (3000 Ci/mmol) in a 50 *μ*l mixture consisting of 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* MgCl$_2$, 0.01% gelatin, and 0.2 m*M* dNTP. For amplification of IGF-II, IGF-IR, cyclin D1, and bcl-2 sequences, 30 cycles of PCR were programmed as follows: denaturation at 95°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min, followed by a final 10 min extension at 72°C using a "Gene Amp" PCR system 480 (Perkin Elmer Cetus, Foster City, CA). PCR of IGF-I comprised 28 cycles with annealing at 60°C. For amplification of GAPDH, the PCR comprised 26 cycles with annealing at 63°C.

The PCR products were then subjected to 5% polyacrylamide gel-electrophoresis, and the radioactivity was measured with a Fuji Bio-Image Analyzer BAS2000 (Fuji Film Co., Ltd., Tokyo). The expression levels of genes were normalized with respect to mRNA expression of GAPDH and expressed as fold expression.

Study patients The study patients were randomly selected from our RNA/DNA bank of breast cancer. We measured mRNA expression of IGF-IR and IGF-II in surgical specimens obtained from 106 primary breast cancer patients; normal mammary gland and cancer tissue from 100 patients and cancer tissue from 6 patients. In addition, IGF-I mRNA expression was measured in normal mammary gland and cancer tissue from 75 patients, and in cancer tissue from another 20 patients. The failure of

measurement in some normal tissues was due to difficulty in isolating sufficient amounts of RNA for experiments. Besides IGFs and IGF-IR, we measured expression of cyclin D1 and bcl-2 mRNA in relation with IGFs.

Statistical analyses Since the expression levels of each gene did not show normal distributions, we used the log-transformed expression levels for statistical analyses. To assess the correlation between two variables, absolute values were used. All statistical analyses were performed using the SPSS statistical package.

RESULTS

Characteristics of study patients The profiles of study patients are summarized in Table I. About 40% of them were postmenopausal, and approximately two-thirds were ER-positive. These proportions were comparable with those in the basic data of all patients in Mammary Surgery at Saitama Cancer Center Hospital. There was no relation-

Table I. Selected Characteristics of Study Subjects by Menopausal Status

	Premenopausal	Postmenopausal
No. of patients	63	43
Age (yrs, range)	44.4 (27–53)	62.0 (48–84)
Stage		
I	18 (28.6)[a]	10 (23.2)
II	37 (58.7)	30 (69.8)
III, IV	8 (12.7)	3 (7.0)
ER status		
(+)[b]	47 (74.6)	27 (62.8)
(−)	16 (25.4)	16 (37.2)
PgR status		
(+)[b]	49 (77.8)	22 (51.2)
(−)	14 (22.2)	21 (48.8)
BMI (kg/m², mean±SD)	22.7±3.13	23.4±2.55
(range)	(15.4–33.3)	(18.1–32.5)
No. of parity		
0	9 (14.3)	5 (11.6)
1–3	41 (65.1)	21 (48.8)
≥4	13 (20.6)	17 (36.6)
Age at first birth		
Nulliparous	10 (15.9)	8 (18.6)
<25	22 (34.9)	8 (18.6)
25–30	29 (46.1)	22 (51.2)
≥31	2 (3.1)	5 (11.6)
Family history of breast cancer[c]		
(+)	9 (14.3)	7 (16.3)
(−)	54 (85.7)	36 (83.7)

a) Values in parentheses are percentages.
b) Values of ≥5 fmol/mg protein are categorized positive.
c) Incidence of breast cancer among relatives within the third degree of relationship by blood.

ship between BMI and status of ER or PgR among premenopausal patients. However, the levels of ER and PgR were positively associated with increasing BMI among postmenopausal patients (data not shown).

Expression of IGF-I, IGF-II, and IGF-IR in normal mammary gland in relation to BMI In this study, we first examined mRNA expression of various genes such as *BRCA1*, *erbB2*, *cyclin D1*, *cyclin E*, and *bcl-2* other than *IGF-IR*, *IGF-I* and *-II* in breast cancer tissues and normal mammary tissue. Among the genes examined, we found an association of BMI with the expression of IGF-II and IGF-IR. Specifically, to study the molecular significance of increased BMI as a potent risk factor in postmenopausal breast cancer, we examined the expression of IGF-I, IGF-II, and IGF-IR in normal mammary gland of premenopausal and postmenopausal patients in association with BMI, which was categorized by tertiles in three groups (round numbers were used for division): low (<21 kg/m²), medium (21–24 kg/m²), and high (>24 kg/m²) (Fig. 1). The expression levels of IGF-IR and IGF-II mRNA show a positive association with BMI among postmenopausal patients. The expression levels of IGF-IR and IGF-II in high BMI subjects were significantly higher than those in low BMI subjects (Student's t test, $P=0.011$ and 0.014, respectively; for trend, $P=0.008$ and 0.022, respectively). These results imply that postmenopausal women with increased body weight have an increased mitogenic stimulus of IGF-II via IGF-IR in their breast epithelium. However, no significant association with BMI was found among premenopausal patients.

In addition, we observed a significant and positive association of IGF-II with IGF-IR in normal mammary gland regardless of menopausal status (Spearman's correlation coefficient of 0.52 at $P<0.001$), although no association between IGF-IR and IGF-I was observed in normal mammary gland.

Expression of IGF-I, IGF-II, and IGF-IR in breast cancer tissue in relation to BMI To find the molecular interpretation of how BMI is associated with malignant progression of breast cancer, we next examined the association of BMI with mRNA expression of IGF-I, IGF-II, and IGF-IR in breast cancer tissue (Fig. 2). The expressions of these genes showed a similar pattern to these in normal mammary tissue. However, the difference between high and low BMI patients was shown only in IGF-IR mRNA among postmenopausal patients (Student's t, $P=0.076$; for trend, $P=0.067$). In premenopausal patients, no consistent or significant association with BMI was observed. Some significant correlations among IGF-I, IGF-II, and IGF-IR were also observed regardless of menopausal status: Spearman's correlation coefficient was 0.53 ($P<0.01$) between IGF-II and IGF-I, and 0.41 ($P<0.01$) between IGF-II and IGF-IR, with no significant correlation between IGF-I and IGF-IR.

Association of mRNA expression of IGF-I, IGF-II, and IGF-IR with the expression of cyclin D1 and bcl-2 The signaling pathway through IGF-IR involves cell responses, specifically cell proliferation and antiapoptosis, as shown by *in vitro* studies,[17, 18, 26] although no clear evidence was found in human breast cancer studies. Then, we further investigated the association of IGFs and IGF-IR mRNA expression with the expression of other genes previously mentioned. We found that the expression of IGF-IR was closely associated with that of cyclin D1 in both normal mammary gland ($n=104$, $r=0.60$, $P<0.001$; n, sample size; r, Spearman's correlation coefficient) and cancer tissue ($n=100$, $r=0.50$, $P<0.001$; Fig. 3), and with that of bcl-2 in cancer tissue regardless of menopausal status ($n=34$, $r=0.77$, $P<0.001$; Fig. 4). These results imply that increased expression of IGF-IR may result in modulation of the cell cycle and apoptosis.
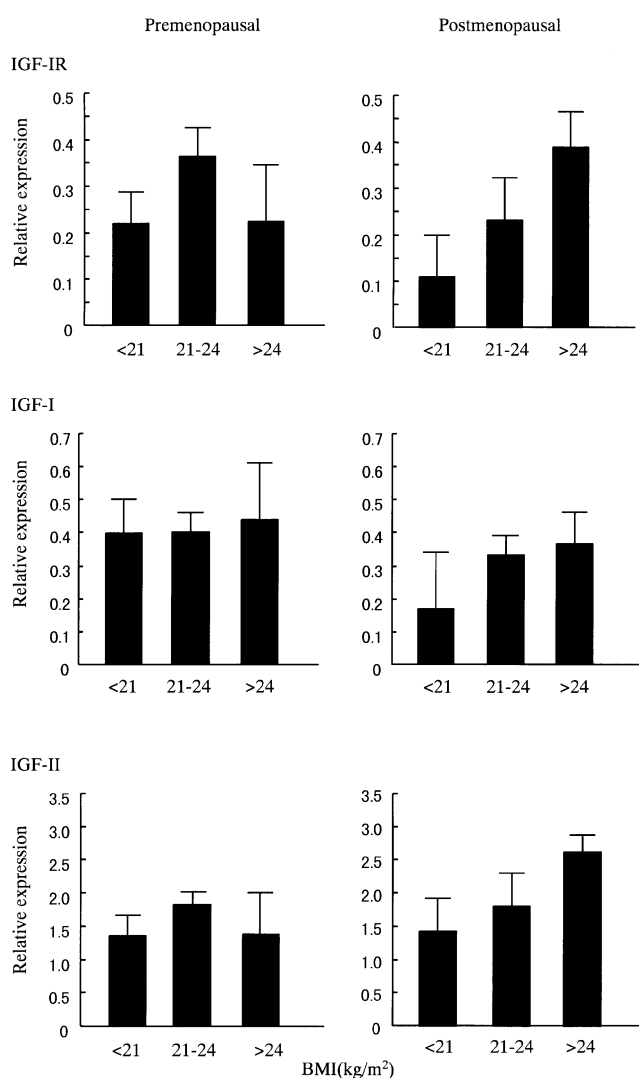

Fig. 1. Geometric means of relative expression levels of IGF IR, IGF-II, and IGF-I mRNAs in normal mammary gland by BMI. Horizontal bars show SEs of the mean. BMI is divided into three categories: <21, 21–24, >24 kg/m². In premenopausal mammary gland, no significant association was found between the expression levels of these genes and BMI. The expression levels of IGF-IR and IGF-II mRNA were positively and significantly associated with BMI in postmenopausal mammary gland.
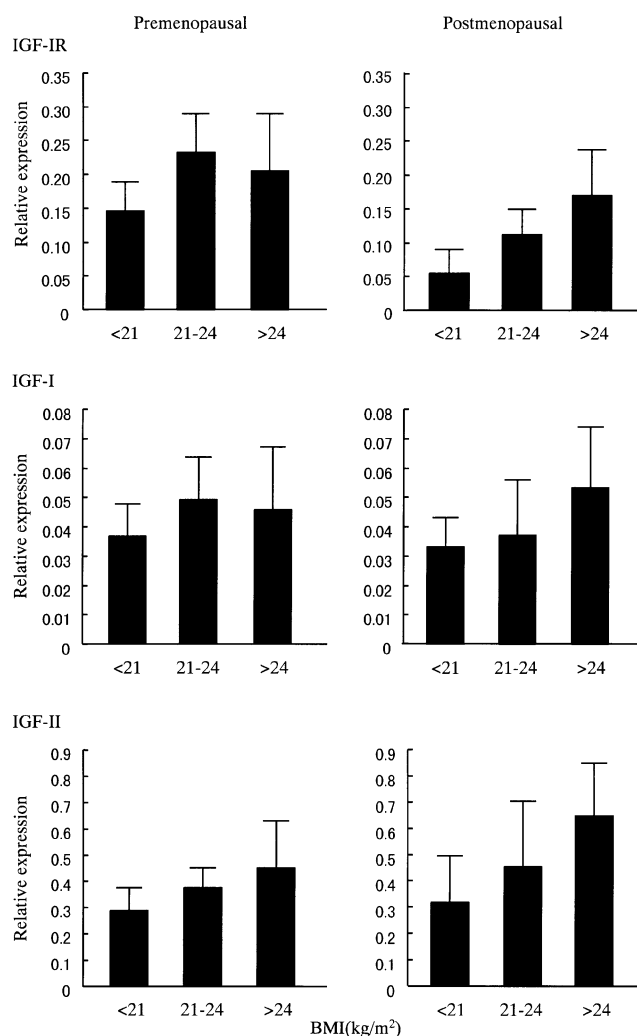
Fig. 2. Geometric means of relative expression levels of IGF IR, IGF-II, and IGF-I mRNAs in breast cancer tissue by BMI. Horizontal bars show SEs of the mean. BMI is divided into three categories: <21, 21–24, >24 kg/m². In postmenopausal breast cancer, expression of IGF-IR mRNA was positively associated with BMI, although no consistent association was found in premenopausal breast cancer.

When we divided the patients by menopausal status (excluding perimenopausal ones and those who had undergone oophorectomy), IGF-IR showed a significant association with cyclin D1 in normal mammary gland of both premenopausal ($n=53$, $r=0.59$, $P<0.001$) and postmenopausal patients ($n=36$, $r=0.40$, $P=0.02$) and also in cancer tissue of both premenopausal ($n=57$, $r=0.55$, $P<0.001$) and postmenopausal patients ($n=36$, $r=0.77$, $P<0.001$). However, BMI did not show a significant association with cyclin D1 in premenopausal ($r=0.04$, $P=0.8$) and postmenopausal patients ($r=0.24$, $P=0.2$), implying that BMI was not directly associated with the expression of cyclin

D1. The association between BMI and bcl-2 could not be examined by menopausal status, due to the small number of study patients.

DISCUSSION

We have been investigating the association of gene expression with epidemiological factors in breast cancer, using our RNA/DNA breast cancer bank.[28, 30] In this paper, we show that increased BMI is positively associated with increased mRNA expression of IGF-IR and IGF-II in normal mammary gland of postmenopausal women. It is of much interest that this association was not found among premenopausal women, a result consistent with the epidemiological finding that increased BMI is a potent risk factor for postmenopausal, but not premenopausal breast cancer. In addition, BMI shows a positive association with increased mRNA of IGF-IR in cancer tissue, explaining in part why increased BMI is associated with a poorer prognosis for breast cancer. These data suggest that postmenopausal women with increased body weight may undergo increased mitogenic effects of IGF-II, and that increasing IGF-IR among these women results in increased sensitivity to the stimulus. Moreover, a close association of the expression of IGF-IR with cyclin D1 and bcl-2 suggests that increased IGF-IR, which is possibly a result of increased BMI, may modulate the cell cycle and apoptosis. Our results thus indicate that increased BMI influences the genesis and progression of breast cancer through increased expression of IGF-IR and IGF-II.

It has been proposed that the association between increased BMI and postmenopausal breast cancer is due to increased peripheral production of estrogen by aromatase
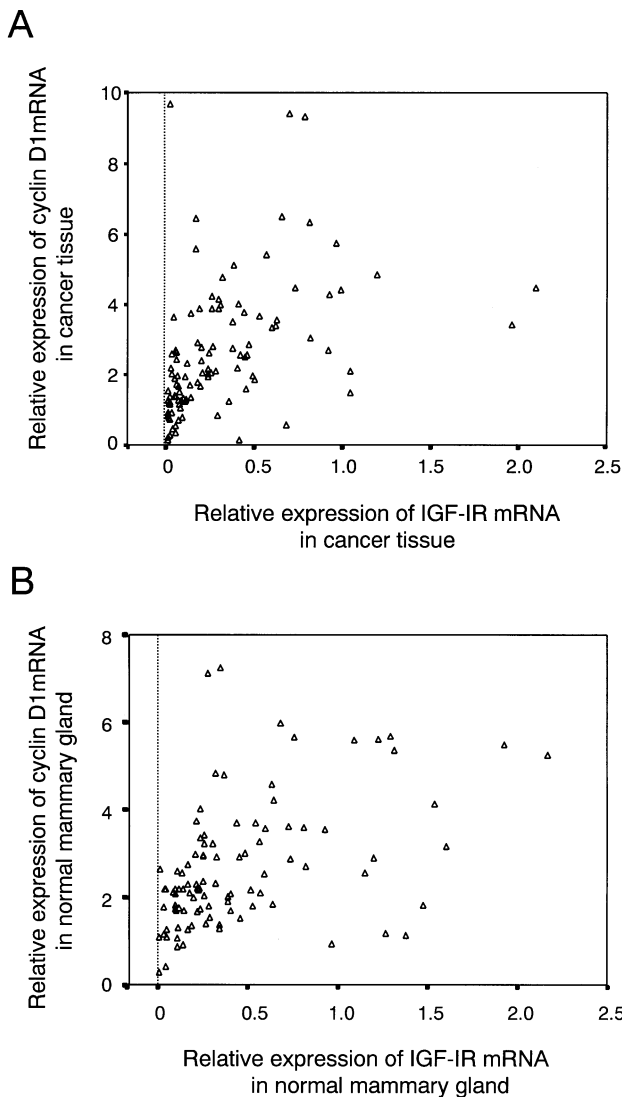

Fig. 3. Correlation between cyclin D1 and IGF-IR mRNAs expression. A, breast cancer tissue, $n=104$, $r=0.60$, $P<0.001$; B, normal mammary gland, $n=100$, $r=0.50$, $P<0.001$. n, sample size; r, Spearman's correlation coefficient.
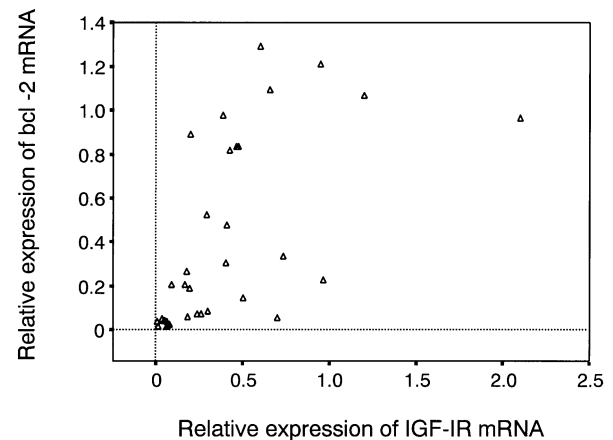

Fig. 4. Correlation between IGF-IR and bcl-2 mRNA expression in breast cancer tissue. n, sample size; r, Spearman's correlation coefficient. $n=34$, $r=0.77$, $P<0.001$.

among obese women. However, this seems not to be the case, at least among Japanese women. Japanese women generally tend to have smaller BMI than Caucasians, and serum estrogen levels among Japanese women are much less than those among Caucasians, with levels among Japanese premenopausal women comparable to those among Caucasian postmenopausal women. We examined the concentration of estradiol in sera and breast tissue of postmenopausal breast cancer patients and found that 67.6% and 89.5% of the patients were below the limits of detection (10 pg/ml and 100 pg/mg, respectively). In brief, no significant association with their BMI was found.

Production of IGF-I and -II is induced by growth hormone and insulin: dietary restriction suppresses the synthesis of both IGF-I and -II at the transcriptional level in laboratory studies using animals[31, 32]; IGF-I is more sensitive to acute dietary restriction than IGF-II, while chronic dietary restriction generates a significant decrease in IGF-II.[31] Locally produced IGF-II, which causes mitotic stimulation of the cells in an autocrine and a paracrine manner, is important, because of low synthesis in the liver among adults; and circulating IGF-I is more important than autocrine or paracrine IGF-I. In this study we examined mRNA expression of IGF-I and -II in breast epithelium or cancer tissue, since interindividual differences in mRNA levels were much larger than those in serum concentration, and since autocrine or paracrine IGF-II in breast tissue was our principal interest. Although we found that transcription of IGF-II within breast tissue was associated with increased host BMI, further study will be required to determine the contribution of circulating IGF-I and -II, which showed little association with BMI but was closely related to the development of breast cancer.[33, 34]

Transcription of the *IGF-IR* gene is known to be regulated by various transcription factors, such as AP-2, Sp1, c/EBP, WT1 and, indirectly, p53, although there is no clear indication as to which was responsible for the observed association with BMI in this study.[35–37] A mechanistic study of this association in future, including cross talk between IGF and ER signaling, may reveal why the association with BMI was found only among postmenopausal women, not premenopausal ones, even though the association of IGF-IR with cyclin D1 and bcl-2 was observed regardless of menopausal status.

To conclude, our results provide not only a mechanistic interpretation of the epidemiological finding, i.e., increased BMI as a positive modifier of IGF-IR expression and locally expressed IGF-II, but also a possible molecular target for prevention of breast cancer, i.e., a negative modification of IGF-IR and IGF-II, possibly through development of chemopreventives.

ACKNOWLEDGMENTS

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