may also have predisposed our patients to the development of prostate cancer. We and others have shown a significant association between low serum testosterone levels and high Gleason score,[16,17] advanced pathologic stage,[18,19] and poor outcome.[20] Moreover, recent results from the Prostate Cancer Prevention Trial suggest that while inhibiting the conversion of testosterone to the more potent dihydrotestosterone by finasteride treatment reduces the number of prostate cancer cases, it also increases the risk of high-grade cancer.[21] Recently, a large multicenter longitudinal study evaluating risk factors of fractures (the Osteoporotic Fractures in Men: MrOS Study) disclosed that a significant inverse relationship was observed for total body BMD with high-grade prostate cancer.[22] Notably, in our study, the Gleason scores correlated inversely with BMD (Fig. 2A,B), which is consistent with the observations described earlier. BMD has been regarded as a possible surrogate marker for lifetime exposure to endogenous sex hormones, insulin-like growth factor I, and calcium intake.[22,23] The relationships between hormonal levels, BMD, and prostate cancer carcinogenesis and malignant potential may be further complicated by racial differences, which have been highlighted by our observation that western and Asian (Japanese) men with prostate cancer differ markedly in the prevalence of osteoporosis. Further research comparing the BMDs and occurrence and characteristics of prostate cancer in these different populations is needed.

In addition, it should be noted that the serum levels of these bone markers in patients with prostate cancer are affected by 2 different influences, namely, the occurrence and progression of bone metastasis, and treatment with ADT. Patients with prostate cancer are frequently subjected to both influences simultaneously. Two recent studies that measured these bone resorptive markers of zoledronic acid–treated prostate cancer patients found that the NTx serum levels rapidly decreased after the zoledronic acid–induced prevention of bone resorption started, whereas the ICTP levels did not alter.[24,25] Thus, there might be a discrepancy between these markers. To provide valuable information regarding both the treatment-induced and disease-dependent bone loss of patients with prostate cancer, further investigation of these bone-turnover markers is necessary.

In conclusion, our cross-sectional study confirmed our previous observation[7] that both hormone-naive and ADT-treated Japanese patients with prostate cancer have similarly low rates of osteoporosis. This is true even for patients treated with ADT for more than 2 years. These findings are quite different from those of studies examining patients in western countries. Genetic and hormonal or other environmental factors may be responsible for differences in not only BMD but also the characteristics of prostate cancer between these populations. To confirm these differences in BMD and the occurrence and characteristics of prostate cancer in these different populations, larger scale and prospective studies will be necessary.

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EDITORIAL COMMENT

The adverse metabolic effects of androgen deprivation therapy (ADT) in men with metastatic prostate cancer are clear. This study by Wang et al discusses the relationship between ADT and bone mineral density (BMD) in Japanese men with prostate cancer, a group previously reported to have a lower incidence of baseline and ADT-induced osteoporosis compared with Caucasians.[1] Yuasa et al[2] provided an interesting article that may lead to additional studies to understand the impact of prostate cancer and its treatment on bone metabolism. This article should also serve as a cautionary note about the appropriate use of ADT for prostate cancer and the use of biomarkers in clinical trials.

To assess the clinical applicability of data in this retrospective study, it is necessary to clearly understand the patient characteristics. As we try to minimize use of ADT due to adverse effects, only 40% of patients receiving ADT in this study had metastatic disease to the bone, the most frequent site of metastatic disease. It would be informative to know why the other 60% of patients were receiving ADT. Was this for metastatic disease to lymph nodes? With adjuvant radiation for locally advanced disease? For an increasing PSA without evidence for metastatic disease? Did patients without metastatic disease to bone have negative bone imaging or was presence of metastatic disease not documented? Each of these populations has a much different likelihood for disease- or treatment-related changes in bone metabolism and, therefore, BMD due to differences in the duration of disease or treatment.

The measurement of markers of bone metabolism in this study adds more confusion than insight and points to the uncertainty associated with using unvalidated markers in clinical trials. There are many markers for bone turnover, including total and bone-specific alkaline phosphatase (tALP and bALP), cross-linked N- and C-terminal telopeptides of type-I collagen (NTx and CTx), amino-terminal procollagen propeptides of type-I collagen (PINP), and C-terminal telopeptides of type-I-collagen (ICTP). It is unclear why NTx and ICTP were chosen for this study. Both NTx and ICTP are appropriately described in the results as measures of bone resorption with a significant increase in ICTP, but not NTx in patients with bone metastasis receiving ADT, purportedly due to a proposed differential effect of ADT and progression of metastatic disease on bone markers. It is not clear why one marker of resorption would be influenced differently than the other. More importantly, the effects of ADT on both NTx and ICTP in patients with bone metastasis would be significantly different from hormone-naive patients with less variation in the serum NTx data.

Differences in the effects of ADT on BMD in Japanese and Caucasian men with prostate cancer may provide a unique opportunity to understand the mechanisms by which hormonal therapies affect bone metabolism. In vitro and clinical trials, with carefully selected markers of bone metabolism, comparing the effects of ADT or bisphosphonates on bone metabolism in these patients may uncover differences in cellular receptors or cell signaling pathways, which could be explored as future therapeutic targets.

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Clinical Significance of Polymorphism and Expression of Chromogranin A and Endothelin-1 in Prostate Cancer

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Abbreviations and Acronyms

BPH = benign prostatic hyperplasia
BPHcont = nonprostate cancer specimen BPH region
BPHpca = prostate cancer specimen BPH region
CHGA = chromogranin A
ET = endothelin
IHC = immunohistochemistry
LD = linkage disequilibrium
PCapca = prostate cancer region
PCR = polymerase chain reaction
PSA = prostate specific antigen
RRP = radical retropubic prostatectomy

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Purpose: We investigated the clinical significance of chromogranin A and endothelin-1 polymorphism and expression in prostate cancer.
Materials and Methods: We analyzed 2 *CHGA* polymorphisms by polymerase chain reaction-restriction fragment length polymorphism in DNA samples of 435 patients with prostate cancer and 316 age matched male controls. Chromogranin A and endothelin-1 expression was evaluated by immunohistochemistry in prostate specimens of 114 men with prostate cancer who underwent radical retropubic prostatectomy and in 27 with bladder cancer who underwent radical cystectomy and served as controls.
Results: For the *CHGA* Glu264Asp polymorphism men with the *GG* genotype were at 2.05 times higher risk for prostate cancer than men with the *CC* genotype ($p = 0.014$). In men with prostate cancer higher chromogranin A immunohistochemistry grade was associated with higher stage and higher Gleason score ($p = 0.011$ and 0.044, respectively). Multivariate analysis showed that chromogranin A immunohistochemistry grade was an independent variable for predicting biochemical failure after radical prostatectomy ($p = 0.023$). Higher endothelin-1 expression was observed in prostate cancers ($p = 0.011$), especially those with a higher Gleason score ($p = 0.042$). There was no significant relationship between chromogranin A polymorphisms, and chromogranin A and endothelin-1 expression.
Conclusions: Polymorphism and expression of chromogranin A and endothelin-1 have clinical significance in prostate cancer. Chromogranin A expression was an independent predictor of biochemical failure after prostatectomy in patients with localized prostate cancer.

Key Words: prostate; prostatic neoplasms; polymorphism, genetic; chromogranin A; endothelin-1

NEUROENDOCRINE cells have an important role in normal prostates and BPH as well as in primary and metastatic prostate cancer.[1,2] Of the biogenic amines and neuropeptides secreted by neuroendocrine cells CHGA is a candidate marker for diagnosing and predicting the prognosis of prostate cancer. Patients with prostate cancer have significantly higher serum CHGA than those with BPH and controls.[2] A group reported that CHGA protein expression determined by IHC is a useful prognostic marker of biochemical failure after radical prostatectomy.[3] To date only 1 group

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has performed IHC analysis and found higher CHGA expression in benign epithelial cells adjacent to prostate cancer lesions than in the BPH region.[4] On the other hand, CHGA polymorphisms can influence CHGA expression, which eventually affects baseline blood pressure,[5] but the relationship between CHGA polymorphisms and prostate cancer remains unclear.

ETs, which are endogenous small peptides secreted by endothelium, exert paracrine and autocrine effects through cell surface receptors and influence cellular processes, such as angiogenesis, cellular proliferation, and tissue repair and development.[6–8] Plasma ET-1 levels in patients with hormone refractory, metastatic prostate cancer are higher than in patients with organ confined prostate cancer or controls.[9] Another IHC study showed ET-1 over expression in cases of advanced prostate cancer and high grade prostatic intraepithelial neoplasia.[10] Recently CHGA and ET-1 interaction was reported in a group of twins as well as in vitro experiments.[11] The study showed that polymorphisms in the CHGA promoter region are associated with serum ET-1 and CHGA stimulated ET-1 secretion in endothelial cells in a dose dependent manner. To our knowledge the association between CHGA and ET-1 in prostate cancer has not been assessed.

We analyzed 7 polymorphisms in the promoter region and the Glu264Asp polymorphism in exon 6 of *CHGA* in a Japanese population to evaluate the relationship to prostate cancer risk and clinical characteristics. We evaluated CHGA and ET-1 protein expression to determine whether they are related to localized prostate cancer pathological features and treatment outcomes. Also, we assessed the relationships among the *CHGA* genotypes, CHGA protein expression and ET-1 protein expression.

MATERIALS AND METHODS

Subjects

A total of 751 men, including 435 with prostate cancer and 316 controls, were enrolled in this study. All patients with prostate cancer were diagnosed at Akita University Medical Center, Kyoto University affiliated hospital and related community hospitals. They were pathologically diagnosed using specimens obtained from transrectal needle biopsy or transurethral prostate resection due to lower urinary tract symptoms. Prostate cancer clinical or pathological stage at diagnosis was determined by reviewing the medical records based on the TNM system. Prostate cancer was classified as stage A—T1a-bN0M0, stage B—T1c-2N0M0, stage C—T3-4N0M0 and stage D—T1-4N1M0-1 or T1-4N0-1M1 by the modified Whitmore-Jewett system. Controls were native Japanese men older than 60 years who had undergone health inspection at a community hospital.

IHC was done in prostate specimens from 114 men with stage T2-4 prostate cancer who underwent RRP and in BPH specimens from 27 who underwent radical cystectomy for bladder cancer. Since endocrine therapy may affect the number of neuroendocrine cells, patients with prostate cancer treated with endocrine therapy before RRP were excluded from analysis.[12] Clinical information was reviewed in the medical records. DNA and prostate specimens were collected after obtaining informed consent with approval from the institutional ethics committee.

CHGA Polymorphism Genotyping

We selected 7 polymorphisms in the *CHGA* promoter region for LD analysis. DNA direct sequencing was done in 200 samples to analyze the genotypes of those polymorphisms. The Appendix lists PCR primer sequences. Genotype data were imported into Haploview, version 3.32 (Daly Laboratory, Board Institute, Cambridge, Massachusetts) to test LD among polymorphisms in the *CHGA* promoter region. D′ greater than 0.8 was considered a strong LD.

Finally, we analyzed 2 *CHGA* polymorphisms, including rs9658635 in the promoter region and Glu264Asp in exon 6, using certain primers (table 1). After confirming successful PCR amplification each product was digested at 37C overnight with 5 U *Bcc* I or *BfuC* I restriction enzymes (New England Biolabs, Beverly, Massachusetts). For the rs9658635 polymorphism restriction fragments were 114 and 21 bp for the *T* allele, and 135 bp for the *C* allele. For the Glu264Asp polymorphism restriction fragments were 129 and 106 bp for the *G* allele, and 235 bp for the *C* allele. To avoid genotyping errors caused by incomplete digestion or other technical failures we repeated the experiment at least twice for all samples and compared the genotype with the DNA sequencing results in 100 randomly selected samples.

Table 1. *PCR primers*

	Reference Single Nucleotide Polymorphism	
	rs9658635	rs9658655
Polymorphism	T-415C	Glu264Asp
Primers	Forward-5′ CCTAGATATTGGAGAGAGCCATGAGTGA 3′ Reverse-5′ CCATGTGTACTGAGGTCCCTGGCAG 3′	Forward-5′ AGGGTGGCAGGCAAAGAG 3′ Reverse-5′ AAGGTGGAATGAGGTTATGG 3′
Length (bp)	135	235
Enzyme	*BccI*	*BfuCI*
Fragments (bp)	21 + 114	106 + 129

IHC Staining and Evaluation

We performed IHC staining for CHGA and ET-1 using a certain protocol. Briefly, deparaffinized, rehydrated sections were steamed for 20 minutes to enhance antigen retrieval. Immunohistochemical labeling with mouse anti-human CHGA antibody (DakoCytomation, Glostrup, Denmark) (×800) or ET-1 antibody (Alexis Biochemicals, Lausen, Switzerland) (×250) was done overnight at 4C. Slides were labeled with the anti-mouse EnVision™+ system labeled with horseradish peroxidase for 30 minutes. The liquid DAB+ Substrate-Chromogen System (DakoCytomation) was applied at room temperature for 30 minutes. Slides were counterstained with hematoxylin solution for nuclear staining. Specimens were examined by 2 independent researchers blinded to sample background data.

CHGA positive stained cells were counted in 10 high power visual fields at 200× magnification to determine which had the most positive cells (fig. 1, *A*). Since the number of CHGA positive cells in the BPH region was greatly different than that in the prostate cancer region, CHGA positive cells in 3 regions were counted, including BPHcont, BPHpca and PCapca. Counting was done 3 times per sample and the mean was used for statistics. The mean value of each sample was categorized as grade 1—less than 10, grade 2—10 to 29 and grade 3—30 or greater for the prostate cancer region. Since neuroendocrine cells are consistently found in the periurethral ducts and verumontanum,[13] those regions were excluded from counting.

Cytoplasmic ET-1 staining intensity was scored on a semiquantitative scale as 1—weak, 2—moderate and 3—strong (fig. 1, *B*). The percent of cytoplasmic ET-1 positive cells was divided into 4 groups, including 1—less than 25%, 2—25% to 50%, 3—50% to 75% and 4—greater than 75%. Total immunoreactivity grade was calculated by multiplying the 2 scores[14] and defined as grade 1—6 or less, grade 2—8 and grade 3—greater than 8.

Statistical Analysis

All data were entered into an Access® database and analyzed using Excel® 2007 and SPSS®, version 16.0J. We examined differences in mean age in the 3 groups using the independent t test. Hardy-Weinberg equilibrium analysis was done to compare observed and expected genotype frequency using the Pearson chi-square test. We used binary logistic regression to assess the association between prostate cancer risk and genotypes by calculating the OR and 95% CI. We hypothesized that the *C* allele of the rs9658635 polymorphism would be an inherent genetic risk factor for prostate cancer and prostate cancer progression. Statistical modeling was done independently on the relative risk of the *CC* or *CT* genotype against the *TT* genotype for rs9658635 using the logistic regression model adjusted by age. For Glu264Asp the *G* allele was hypothesized as an inherent genetic risk factor for prostate cancer and prostate cancer progression.

We used 1-way ANOVA to compare the number of CHGA IHC positive cells among the 3 groups and Kendall's τ-b rank correlation coefficients to examine the relationship between IHC grade and Gleason score or clinical stage. The biochemical failure-free interval was defined as the time from the date of RRP to the date when PSA increased to more than 0.4 ng/ml. We estimated relationships between polymorphisms or IHC grade and biochemical failure-free survival in stage T2-4 prostate cancer cases by the Kaplan-Meier method and evaluated them by the log rank test. The Cox multivariate proportional hazards model was used for multivariate analysis. We examined relationships between polymorphisms and IHC grades in patients with prostate cancer using Fisher's exact test. All statistical tests and p values were 2-tailed with results considered significant at $p < 0.05$.

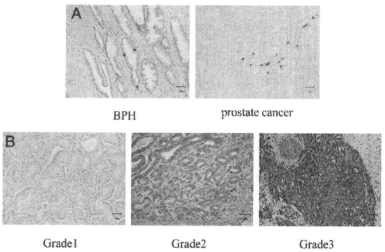

Figure 1. IHC in BPH and prostate cancer regions. *A*, CHGA cytoplasmic staining pattern. *B*, representative ET-1 IHC stains of different grades.

RESULTS

Characteristics

Mean age ± SD in patients with prostate cancer and male controls was 70.28 ± 7.43 and 69.46 ± 7.22 years, respectively ($p = 0.289$). Stage was A to C, D1 and D2 in 10, 191, 83, 25 and 126 patients with prostate cancer, respectively. In the prostate cancer group Gleason score was less than 7, 7, greater than 7 and unavailable in 14, 202, 164 and 55 patients, respectively.

CHGA Associations

Polymorphism genotypes vs prostate cancer risk and clinicopathological factors. Genotype distributions in all groups were consistent with Hardy-Weinberg equilibrium. Since more than 90% of D′ values in the 7 polymorphisms in the *CHGA* promoter region equaled 1 (fig. 2), the rs9658635 polymorphism, which was reported to be associated with CHGA expression,[5] was chosen as a representative polymorphism for further analysis. Statistical analysis of genotype frequency showed no relationship between the rs9658635 polymorphism and the prostate cancer risk ($p > 0.05$, table 2). For the Glu264Asp polymorphism we found a significantly increased prostate cancer risk in men with the *GG*

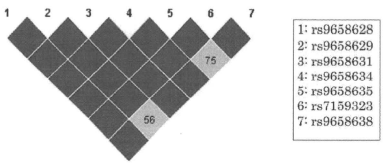

Figure 2. There were strong LDs among 7 CHGA promoter region polymorphisms. Red diamonds indicate D′ = 1. Gray diamonds indicate D′ less than 1.

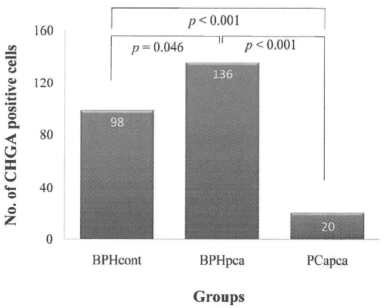

Figure 3. There were significant differences among mean number of CHGA IHC positive cells in BPHcont, BPHpca and PCapca.

genotype and the *GC* genotype than in those with the *CC* genotype (OR 2.05, 95% CI 1.16–3.63; p = 0.014 and OR 1.97, 95% CI 1.10–3.52; p = 0.023, respectively, table 2). There was no significant association of the rs9658635 or the Glu264Asp *CHGA* polymorphism with prostate cancer clinical stage or Gleason score (p >0.05).

IHC grade vs prostate cancer clinicopathological factors and prognosis. BPHcont, BPHpca and PCapca showed a mean ± SD of 97 ± 81, 136 ± 109 and 20 ± 48 CHGA IHC positive cells, respectively (p <0.001, fig. 3). Compared with BPHcont BPHpca had more and PCapca had fewer CHGA positive cells (p = 0.046 and <0.001, respectively). In patients with prostate cancer a higher CHGA IHC grade was more often found in those with pT3-4 than pT2 disease (p = 0.011). There was a significant association between CHGA IHC grade and Gleason score (p = 0.044). On univariate analysis a higher probability of biochemical failure after RRP was significantly associated with higher CHGA IHC grade (p = 0.001, fig. 4), higher Gleason score (p = 0.039), higher stage (p = 0.025) and higher PSA at diagnosis (p <0.001). On multivariate analysis CHGA IHC grade was an independent factor predicting possible biochemical failure after RRP (p = 0.023, table 3).

ET-1 IHC Grade

Prostate cancer showed a higher ET-1 IHC grade than BPH (chi-square 9.030, p = 0.011). Of patients with prostate cancer we noted a higher ET-1 IHC grade in those with a higher Gleason score (chi-square 4.149, p = 0.042, fig. 5). There was no statistically significant relationship between ET-1 IHC grade and clinical stage or biochemical failure after RRP (p = 0.661 and 0.230, respectively).

CHGA Polymorphism Genotypes vs CHGA and ET-1

Cross-tabulation results showed no significant association of the *CHGA* rs9658635 or the Glu264Asp polymorphism with CHGA or ET-1 IHC grade (table 4). We found no significant relationship between CHGA and ET-1 expression (p >0.05).

Table 2. *CHGA polymorphisms vs prostate cancer risk and clinicopathological factors*

	Prostate Ca vs Control		Clinical Stage D vs A + B + C		Gleason Score 8 or Greater vs Less Than 8	
Genotype Polymorphism	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
CHGA promoter rs9658635:						
TT	1		1		1	
CT	1.12 (0.81–1.55)	0.502	1.41 (0.87–2.31)	0.167	1.26 (0.79–1.98)	0.331
CC	0.83 (0.55–1.25)	0.363	1.24 (0.65–2.39)	0.512	1.03 (0.56–1.91)	0.914
CT + CC	1.02 (0.76–1.38)	0.892	1.37 (0.86–2.18)	0.187	1.19 (0.77–1.84)	0.424
TT + CT:CC	1.29 (0.89–1.86)	0.185	0.98 (0.55–1.76)	0.954	1.10 (0.64–1.91)	0.729
Exon 6 Glu264Asp:						
CC	1		1		1	
GC	1.97 (1.10–3.52)	0.023	1.80 (0.64–5.10)	0.268	2.98 (0.96–9.22)	0.059
GG	2.05 (1.16–3.63)	0.014	2.11 (0.76–5.87)	0.154	2.62 (0.86–8.04)	0.091
GC + CC	2.01 (1.15–3.51)	0.014	1.98 (0.72–5.43)	0.817	2.77 (0.91–8.37)	0.072
CC + GC:GG	0.86 (0.64–1.15)	0.314	0.80 (0.54–1.20)	0.279	1.02 (0.69–1.51)	0.904

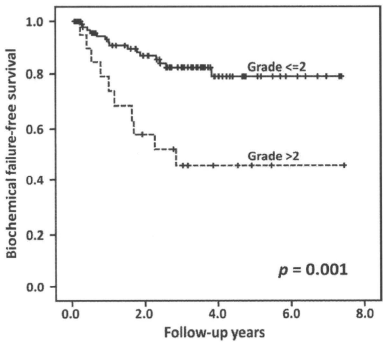

Figure 4. Higher CHGA IHC grade was associated with higher probability of biochemical failure after RRP.

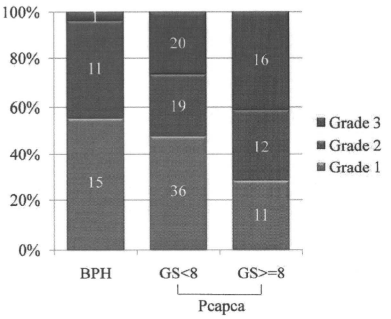

Figure 5. Compared with BPH prostate cancer showed higher ET-1 IHC grade. Higher ET-1 IHC grade was associated with higher Gleason score (*GS*) prostate cancer.

DISCUSSION

We first investigated the influence of CHGA polymorphisms on prostate cancer clinicopathological factors. Results revealed a significant association between the *G* allele of the *CHGA* Glu264Asp polymorphism and the risk of prostate cancer in a native Japanese population, suggesting that the *CHGA* Glu264Asp polymorphism may be a useful marker for estimating the prostate cancer risk. To our knowledge this is the first study to investigate whether *CHGA* gene variants influence prostate cancer. The *G* to *C* allele variant of the Glu264Asp polymorphism caused the 264 amino acid CHGA to change from glutamic to aspartic acid. Pancreastatin, an impairing glucose metabolism peptide of 52 amino acids, is located in this CHGA encoding region.[15] Pancreastatin inhibits the release of glucose stimulated insulin from pancreatic islet β cells.[16] Since insulin has an important role in prostate cancer pathogenesis,[17] it is reasonable that the *CHGA* Glu264Asp polymorphism affects prostate cancer carcinogenesis through functional alteration of pancreastatin by regulating insulin secretion. Also, the importance of the pancreastatin polymorphism was supported by the observation that the Gly297Ser polymorphism influences glucose uptake.[18] Pancreastatin is a useful prognostic indicator in patients with neuroendocrine tumors[19] but there was no association between the CHGA Glu264Asp polymorphism and prostate cancer prognosis.

The CHGA rs9658634 polymorphism is reportedly associated with serum CHGA and the ET-1 level.[5,11] However, the CHGA polymorphism showed no relationship with CHGA or ET-1 IHC expression in our study, which could have been due to several reasons. 1) The different methods of measuring CHGA expression may have led to different results. Since CHGA is secreted by other tissues as well as the prostate, serum CHGA represents total CHGA expression in the whole body. We used prostate surgical specimen IHC grade instead of the serum level to more specifically reflect prostate CHGA expression in the prostate. In support of our findings another research group found no correlation between serum CHGA and immunohistochemical results.[20] 2) The discrepancy may have been be due to our population. Only men older than 60 years with prostate cancer were enrolled in our study

Table 3. *Biochemical failure and clinical factor Cox proportional hazards model*

	Univariate		Multivariate	
Clinical Factors	HR (95% CI)	p Value	HR (95% CI)	p Value
CHGA IHC grade greater than 2 or not	3.585 (1.609–7.987)	0.002	2.713 (1.149–6.407)	0.023
Gleason score 8 or greater or not	2.237 (1.020–4.908)	0.044	1.512 (0.650–3.517)	0.337
T stage greater than 2 or not	2.422 (1.088–5.394)	0.030	0.969 (0.375–2.504)	0.948
PSA greater than 10 ng/ml or not	5.682 (1.950–16.557)	0.001	4.611 (1.483–14.336)	0.008

Table 4. *CHGA polymorphisms vs CHGA and ET-1 expression*

	CHGA IHC Grade				ET-1 IHC Grade			
	1	2	3	p Value	1	2	3	p Value
rs9658635:				0.964				0.497
TT	29	6	9		19	14	11	
CT	32	10	9		18	14	19	
CC	13	3	3		10	3	6	
Glu264Asp:				0.423				0.272
CC	4	0	0		1	2	1	
GC	27	11	10		17	17	14	
GG	43	8	11		29	12	21	

whereas other studies included subjects without cancer regardless of age or gender. This may result in the lack of a significant relationship between CHGA polymorphisms and expression in patients with prostate cancer.

However, our study shows that in patients with localized prostate cancer and no history of endocrine therapy a higher CHGA IHC grade was associated with worse tumor stage and higher Gleason score. A group also reported that IHC staining for CHGA is significantly associated with Gleason score,[20] although a contradictory result was reported.[21] Furthermore, the Cox multivariate regression model showed that CHGA IHC grade was an independent variable for predicting biochemical failure after RRP. In a study of lymph node positive cases of prostate cancer the investigators found that CHGA expression is associated with biochemical failure after RRP.[22] However, the history of endocrine therapy before RRP, which was associated with CHGA expression,[12] was not controlled in that study. Other studies in D2 prostate cancer cases showed that higher CHGA IHC grade is associated with a worse prognosis.[21,23] Taken together, CHGA IHC grade, which represents neuroendocrine differentiation, could predict the prognosis in patients with prostate cancer.

Compared with BPHcont, the number of CHGA IHC positive cells was higher in BPHpca and lower in PCapca (fig. 3). This agrees with the result that CHGA positive cells had more prominent expression in benign epithelial cells adjacent to prostate cancer lesions than in the prostate cancer region.[4] Also, there is a tendency toward a decreased number of neuroendocrine cells in untreated patients with prostate cancer compared with that in patients with BPH and male controls with a normal prostate.[24] Hence, higher serum CHGA in patients with prostate cancer may result from BPHpca, which has many more neuroendocrine cells than in patients with BPH. This indicates that to predict prostate cancer susceptibility more efficiently we should focus on the cancerous region and the adjacent noncancerous region.

Neuroendocrine cells in the BPH region are negative for α-methylacyl coenzyme A racemase while neuroendocrine cells in prostate cancer are positive for α-methylacyl coenzyme A racemase.[25] In vitro cells of the androgen dependent line LNCaP were induced to show neuroendocrine differentiation by androgen deprivation[26] or agents that increase intracellular cyclic adenosine monophosphate.[27] Results indicate that PCapca neuroendocrine cells, which have hormone insensitive characteristics, may differentiate from prostate cancer cells. Patients with the worst prostate cancer stages had a higher CHGA IHC grade, indicating that more prostate cancer cells had transformed into neuroendocrine cells with hormone insensitive characteristics and resulting in a worse prognosis. Whether our patients with hormone refractory prostate cancer had many more hormone insensitive neuroendocrine cells than our patients with localized prostate cancer should be explored in the future.

Serum ET-1 has no value for estimating prostate cancer prognosis.[28] We noted no association of ET-1 expression with clinical stage or biochemical failure after RRP. However, prostate cancer showed significantly higher ET-1 expression than the BPH region and higher ET-1 IHC grade was associated with a higher Gleason score.

CONCLUSIONS

A *CHGA* genetic variant may modify prostate cancer carcinogenesis and CHGA expression may be a useful biomarker to predict the higher malignant potential of localized prostate cancer and biochemical failure after RRP. Thus, results suggest that CHGA is involved in prostate cancer carcinogenesis and progression.

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APPENDIX

DNA Sequencing Primers for CHGA Promoter Region

Fragment No. (polymorphisms)	Reference Single Nucleotide Polymorphism	Primers	
		PCR	DNA Sequencing
1:		Forward-5′ CAGGTTCTCATTTAGGGACA 3′ Reverse-5′ AAAGGTCAGTTTCCTGGTTG 3′	Forward-5′ TTTAGGGACAGGGGTGAGCACAGGT 3′ Reverse-5′ TCAGTTTCCTGGTTGGCTTCCCTT 3′
G-1106A	rs9658628		
A-1018T	rs9658629		
T-998G	rs9658631		
2:		Forward-5′ CATCAGTTACCTGTCAAGTGCGT 3′ Reverse-5′ CCCCGTGCTATTTTTCCTAAGT 3′	Forward-5′ TGTCAAGTGCGTTTCCTCTGT 3′ Reverse-5′ TTCCTAAGTGCCCTCTGCCT 3′
G-462A	rs9658634		
T-415C	rs9658635		
3:		Forward-5′ GCCCAGGGACACAAGGCAAAT 3′ Reverse-5′ TCGGCGTGCGTCCGTCTGTC 3′	Forward-5′ CACCTCTTGGAAACCAGATACC 3′ Reverse-5′ TGCGTCCGTCTGTCGGTCGATG 3′
C-89A	rs7159323		
C-57T	rs9658638		

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Prospective randomized phase II study determines the clinical usefulness of genetic biomarkers for sensitivity to primary chemotherapy with paclitaxel in breast cancer

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In patients with breast cancer, taxane as well as anthracycline play central roles in systemic chemotherapy. By evaluating the pathological response, we can gauge sensitivity to primary chemotherapy. However, biomarkers that would predict a response to taxane have not yet been established. We conducted a prospective randomized trial to evaluate whether selecting patients using sensitivity testing based on the gene expression of the tumor might enhance the probability of the pathological response. Five genes were identified as biomarkers derived from a microarray of DNA gene profiles from microdisected breast tumors. In the experimental arm (B1), 12 cycles of weekly paclitaxel, 80 mg/m^2, were preoperatively given when the sensitivity test was positive and therefore judged to be sensitive to paclitaxel. When the test was negative, meaning insensitive to paclitaxel, four cycles of FEC100 were given (arm B2). In the control arm (A), paclitaxel was administered weekly without the use of the sensitivity test. A total of 92 patients were enrolled and 86 patients were analyzed. The pathological response rate (pRR) of each arm was 36.4% in B1 (expected sensitive to paclitaxel), 21.1% in A (control) and 12.5% in B2, respectively. Weekly paclitaxel-treated patients selected by the sensitivity test did not enhance the pRR. The study failed to validate sensitivity testing using five gene expressions for primary chemotherapy with paclitaxel in patients with breast cancer. However, this study suggests that a randomized phase II study is a robust tool for obtaining a rapid conclusion on the usefulness of biomarkers and could be the foundation for further large clinical trials. (*Cancer Sci* 2011; 102: 130–136)

Trastuzumab, a molecular targeted agent, has greatly improved the survival rate in patients with breast cancer.[1] Trastuzumab binds human epidermal growth factor receptor type 2 (HER2) and downregulates cell proliferation signaling. Trastuzumab enriches its activity by selecting patients with HER2-overexpressed breast cancer. Biomarkers can both maximize activity and minimize toxicities. Cytotoxic agents such as taxane or anthracycline also play a crucial role in systemic chemotherapy for breast cancer.[2] To date, no specific biomarker of cytotoxic chemotherapeutic agents has been established.

Primary chemotherapy with anthracycline and taxane is standard care for patients with early-stage breast cancers to obtain breast conservation and survival benefit.[3] Primary chemotherapy informs us of its sensitivity by evaluation of the pathological response. The probability of a pathological complete response (pCR) from a single administration of taxane is no more than 20%.[4] In our experience, primary treatment with paclitaxel weekly produced a 7% pCR with complete disappearance of intraductal lesions and a 30% pathological response with more than two-thirds reduction in invasive lesions.[5] Taxane induces microtubule bundling, formation of multipolar spindles, mitotic arrest and apoptosis. Resistance to taxane derives from overexpression of ATP-binding cassette (ABC) transporter, for example, P-glycoprotein,[6] somatic mutation of β-tubulin,[7] βIII-tubulin isoform[8] or low expression of tubulin-binding protein tau.[9] However, the clinical usefulness of these biomarkers has not been determined. The DNA microarray provides a unique molecular portrait or signature regarding clinical behavior and drug responsiveness.[10–14] The expression pattern of selected genes, if found to be related to the sensitivity of cytotoxic agents, could yield a biomarker to predict the clinical response and outcome. We have developed a sensitivity test using quantitative RT-PCR of five selected genes to predict the response to paclitaxel. Commonly, retrospective studies have been used to find predictive biomarkers, but their level of evidence is low. To our knowledge, there have been few randomized trials directly addressing biomarkers in a prospective fashion.

Therefore, we have conducted a prospective randomized trial on whether the selection of patients using a sensitivity test to predict paclitaxel based on the gene expression of the tumor might enhance the probability of the pathological response. The current study aimed to validate the genetic diagnosis to predict sensitivity in primary chemotherapy with paclitaxel in women with breast cancer.

Materials and Methods

Patients. Eligible patients were women with histologically confirmed invasive carcinomas of the breast with a tumor size 3 cm or more in stages IIA, IIB, IIIA or IIIB (T1-4, N0-1 and M0). All patients were younger than 70 years and had performance status (Eastern Cooperative Oncology Group performance status) 0 or 1; life expectancy 6 months or more; adequate organ function; white blood cell count 4.0×10^9/L or absolute neutrophil count 2.0×10^9/L; hemoglobin 9 g/dL; platelets 100×10^9/L; blood urea nitrogen (BUN) and serum creatinine within normal limits; aspartate transaminase (AST), alanine transaminase (ALT) twice the upper limit of normal; total bilirubin 1.5 mg/dL; and electrocardiography (ECG) within normal limits. Excluded patients were those with

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Name of the trial register: Validation of genetic diagnosis to predict sensitivity in primary systemic chemotherapy with paclitaxel in women with breast cancer.
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Table 1. Five genes identified as biomarkers

Gene ID	Affy probe	GenBank	UniGene	Gene Symbol	Uni-title
03921	223235	NM022138	Hs.487200	SMOC2	Secreted protein, acidic, cystein-rich related modular calcium binding 2
05918	NA	BG928645	Hs.494395	C9orf121	Chromosome 9 open reading frame 121
06334	205009	NM003225	Hs.162807	TFF1	Trefoil factor 1
19403	224968	NM080667	Hs.264208	CCDC104	Coiled-coil domain containing 104
20850	229580	BX097190	Hs.7413	NA	Transcribed locus

NA, not applicable.

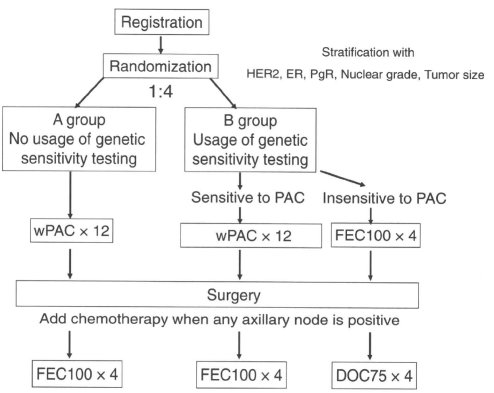

Fig. 1. Study design. Patients were stratified according to the status of human epidermal growth factor receptor type 2 (HER2), estrogen receptor (ER), progesteron receptor (PgR), nuclear grade and tumor size. Patients were randomly assigned to receive arm A or B with a ratio of 1:4. In arm A, patients received primary chemotherapy with paclitaxel without selection by genetic sensitivity testing. For patients in arm B, we used the genetic diagnosis for sensitivity to paclitaxel. In arm B1, patients diagnosed as sensitive to paclitaxel received paclitaxel. In arm B2, patients diagnosed as insensitive to paclitaxel received FEC100. When any axillary node was positive for cancer after curative breast surgery, additional chemotherapy was used. Patients pretreated with paclitaxel received FEC100. Patients pretreated with FEC100 received docetaxel. wPAC × 12, 12 cycles of weekly paclitaxel 80 mg/m^2; FEC100 × 4, four cycles of combination chemotherapy with fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2 and cyrophosphamide 500 mg/m^2 every 3 weeks; DOC75 × 4, four cycles of docetaxel 75 mg/m^2 every 3 weeks.

non-invasive or microinvasive breast cancer, stage IIIC or IV; inflammatory breast cancer; male gender; previous chemotherapy, hormone therapy or radiotherapy; active double cancer; serious complication with infection, cardiac disease, pulmonary fibrosis, interstitial pneumonitis, bleeding, hepatitis type B and its carrier; uncontrolled diabetes; heavy history of drug allergy, history of allergic reaction to drugs using the vehicle cremophor; pregnant, nursing or willing to become pregnant; or otherwise judged inadmissible by the investigators. The research ethics committee of Cancer Institute Hospital approved the study, and all patients gave written, informed consent.

Sensitivity testing. How the sensitivity testing was developed has been described in previous papers.[15,16] Basically, specimens were obtained by core needle biopsy before primary chemotherapy. To minimize the influence of stromal cells, pure populations of tumor cells were collected by laser captured microdissection. After RNA extraction, we performed gene expression profiling of 21 000 genes by DNA microarray to select the candidate genes. Surgically resected primary breast tumors were examined to determine the pathological response to chemotherapy. All clinical and genomic data were entered into an integrated database and analyzed to identify predictive factors. Differentially expressed genes were selected between the paclitaxel-resistant group and the paclitaxel-sensitive group. Then the expression of selected candidate genes was quantified by RT-PCR to confirm the array data and increase reliability. Furthermore, we narrowed the candidate genes down to establish a prediction system based on real-time RT-PCR. Finally, we identified a set of five genes predictive of patient response to paclitaxel in primary chemotherapy (Table 1). Before clinical application, the prediction system was validated retrospectively, revealing that in 51 patients the sensitivity testing using the expression of five genes produced 90% accuracy and a 9.8% error rate.

Study design. To validate the predictiveness of the pathological response by the sensitivity test in primary chemotherapy with paclitaxel, we conducted a prospective randomized trial, as shown in Fig. 1. Patients were stratified according to the status of HER2, estrogen receptor (ER), progesteron receptor (PgR), nuclear grade and tumor size. Participating patients were randomly assigned to receive arm A or B with a ratio of 1:4. For patients in arm A, we did not use the genetic diagnosis for sensitivity to paclitaxel, but they received primary chemotherapy with paclitaxel. For patients in arm B, we did use the genetic diagnosis for sensitivity to paclitaxel. When patients were diagnosed as sensitive to paclitaxel, they received primary chemotherapy with paclitaxel. Patients diagnosed as insensitive to paclitaxel received primary chemotherapy with FEC100.

Unless their disease progressed, patients were treated with 12 weeks of paclitaxel or four cycles of FEC100 and then underwent standard surgery. When any axillary node was positive for cancer, additional chemotherapy was used after surgery. Patients pretreated with paclitaxel received FEC100 and those pretreated with FEC100 (diagnosed as insensitive to paclitaxel) received docetaxel after surgery. In a partial resection of the breast, radiation was performed. If cancer was positive in four or more axillary nodes, prophylactic radiation was performed to the chest and regional nodes. Radiation was applied after completion of chemotherapy. In cases with positive estrogen receptor and/or progesterone receptor, appropriate endocrine

treatment of tamoxifen or aromatase inhibitors was used after completion of chemotherapy. Patients with HER2-overexpressed breast cancer received tri-weekly trastuzumab at a dose of 8 mg/kg followed by 6 mg/kg for 1 year after completion of surgery or postsurgical chemotherapy. Adjuvant trastuzumab was used subsequent to February 2008, which was the approval date in Japan.

Treatment. Paclitaxel was administrated at a dose of 80 mg/m^2 as an intravenous infusion over a period of 1 h every week for 12 weeks. Dexamethasone 10 mg, ranitidine 50 mg and granisetoron 3 mg were given intravenously 30 min before paclitaxel. Diphenhydramine 50 mg was given orally just before infusion. FEC100 consisting of fluorouracil (500 mg/m^2), epirubicin (100 mg/m^2) and cyclophosphamide (500 mg/m^2) was administered intravenously every 3 weeks for four cycles. Dexamethasone 20 mg and granisetoron 3 mg were given intravenously before FEC100. Docetaxel was intravenously administrated at a dose of 75 mg/m^2 for a 1 h infusion every 3 weeks for four cycles. Dexamethasone 8 mg was given as an intravenous infusion on day 1 followed by oral intake on days 2 and 3.

End-points. The primary end-point targeted improvement of the pathological response rate (pRR) as the percentage of patients with grade 2 and 3 as shown by sensitivity testing. The pathological response with grade 2 or 3 was defined as more than a two-third reduction in invasive lesions or complete disappearance of tumors, including intraductal lesions, respectively.[17] Secondary end-points examined the pathological complete response rate (probability of pathological response with grade 3), clinical response rate by the Response Evaluation Criteria in Solid Tumors guidelines (RECIST),[17] breast conservation rate, disappearance rate of axillary node metastasis, distant-metastasis-free survival, disease-free survival and overall survival. Adverse events and laboratory parameters were graded according to the National Cancer Institute, Common Toxicity Criteria, version 3.0.

Statistical analysis. Validity was defined as accuracy of the prediction system for sensitivity testing. Improvement of the pathological response was judged as high accuracy of the prediction system for sensitivity testing. The pathological response rates to paclitaxel in patients who were diagnosed as positive by sensitivity testing were compared with those in patients treated with paclitaxel who did not receive sensitivity testing. The difference in response rate in the two groups was assessed by the Fisher exact test for 2×2 contingency tables. The pathological response rate in the experimental arm was estimated as 80% compared with 30% in the control arm, which was calculated from 29% (15/51) of the pathological response rate in previous unpublished data. A sample size of 21 assessable patients in each arm (A and B1) was required to achieve 90% power with 5% error (two sided). A sample size of arm B (B1 + B2) required 72 ($21 \times 100/29$) patients. The number of cases that dropped out for any reason including inadequate sampling was estimated as 15%. A total of 109 patients were required in the current study. Patients were randomly assigned to receive arm A or B with a ratio of 1:4. An interim analysis was planned when at least 10 pathological assessable patients were obtained in arm B1. Disease-free survival and overall survival were calculated by the Kaplan–Meier method.

Results

Patient characteristics. Ninety-two patients were registered and assessed between February 2006 and February 2009 at the Cancer Institute Hospital. Six patients had too few tumor specimens to evaluate sensitivity testing. Eighty-six patients were randomized. In two patients, we were not able to assess the pathological response in the resected breast tumors, because of progression during primary chemotherapy and a withdrawal of consent to additional post-surgical chemotherapy. A total of 85 patients were assessed for pathological response at surgery. The median follow-up time of patients was 40.0 months, and the range was 17.0–49.8 months. All patients were Japanese women. The demographic characteristics of the present study population are presented in Table 2. The median age was 52.5 years (range, 31–68). Median size of tumor estimated as an invasive lesion was 3.75 cm (range, 3.0–9.9). While 81% of patients were T2, 41% of patients had no clinical axillary lymph node metastasis. Histology showed papillotubular carcinoma (8%), solid-tubular carcinoma (24%) or scirrhous carcinoma (65%). In 24% of patients, we found nuclear grade 3. Estrogen receptor or PgR was positive in 71% or 47% of patients, respectively. Positive HER2 status was defined as immunohistological (Hercep test) score 3+ (>10%) or FISH positive (ratio >2.0). Twenty-one percent of patients were HER2 positive. Intrinsic subtypes were divided as follows. Luminal A was defined as negative HER2 status with ER positive and/or PgR positive. Luminal B was defined as positive HER2 status with ER positive and/or PgR positive. HER2 subtype was positive HER2 status with both ER and PgR negative. Triple negative was HER2 negative, ER negative and PgR negative. Luminal A, Luminal B, HER2 subtype or triple negative was 64%, 8%, 13% or 15%, respectively. The background of arms A and B (B1 + B2) was mostly balanced except for a slight tendency towards more patients with papillotubular carcinoma, HER2 positive or luminal B, and fewer patients with grade 3 in arm A. The background in arms A and B1 was different because of selection by sensitivity testing.

Pathological response and clinical outcome. Interim analysis was performed after 11 patients were assessable for pathological response in arm B1. As shown in Table 3, the patients in arm B1, diagnosed as sensitive to paclitaxel, demonstrated 36.4% (4/11) of the pathological response rate, whereas patients who did not use sensitivity testing of paclitaxel showed 21.1% (4/19). The difference between arms A and B1 was not significant ($P = 0.627$). Since the pathological response rate (36.4%) of the experimental arm (B1) was far below the expected rate of 80% despite achievement with 82% (89/109) of the planned accrual number, the committee decided to terminate the study. In arm B2, the patients who were treated with FEC100 judged as insensitive to paclitaxel showed 12.5% (7/56) of the response rate. A pathological complete response was seen in 3.6% (2/56) of FEC100 (B2), but no complete response in the paclitaxel arms (A1 or B1). Pathological metastasis in resected lymph nodes at surgery was absent in 27% (3/11) of arm B1, in which the mean number of pathological positive nodes was 3.8. In one out of four pathological responders with grade 2 and 3, all axillary nodes disappeared. The clinical response rate of the paclitaxel-sensitive group (B1) was not improved at 55% (6/11) as compared with 53% (10/19) of the control arm (A) or 54% (30/56) in patients who were treated with FEC100 (B2). The breast conservation rate was not improved at 36% in arm B1, compared with arm A (32%) or arm B2 (52%). Disease-free survival and overall survival at 3 years in all patients ($n = 86$) were 81.2% and 94.6%, respectively (Fig. 2). Disease-free survival at 3 years in arms A, B1 and B2 was 72.3%, 62.3% and 87.6%, respectively. Adverse events are summarized in Table 4. One patient, who dropped out after five cycles of preoperative paclitaxel, was excluded to evaluate toxicity. A total of 85 patients were assessed for toxicity. Grade 3 or 4 of adverse events in the preoperative paclitaxel (A + B1) or FEC100 (B2) was 1.0% and 8.1%, respectively. There was no difference in the profile of adverse events between arms A and B (data not shown). No unexpected adverse events were observed.

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Table 2. Patient characteristics

Sensitivity testing	A	B1	B2	Subtotal of patients in B (B1 + B2)	All patients
	Not performed	Performed			
		Sensitive to paclitaxel	Insensitive to paclitaxel		
Treatment	Paclitaxel	Paclitaxel	FEC100		
No. randomized patients	19	11	56	67	86
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Median age	50	57.0	52.0	53.0	52.5
T					
T2	14 (74)	8 (73)	48 (86)	56 (84)	70 (81)
T3	4 (21)	3 (27)	5 (9)	8 (12)	12 (14)
T4	1 (5)	0 (0)	3 (5)	3 (4)	4 (5)
Median size (cm)	3.6	4.2	3.6	3.8	3.75
Range (cm)	3.0–5.7	3.0–5.8	3.0–9.9	3.0–9.9	3.0–9.9
N					
0	7 (37)	4 (36)	24 (43)	28 (42)	35 (41)
1	11 (58)	6 (55)	31 (55)	37 (55)	48 (56)
2	1 (5)	1 (9)	1 (2)	2 (3)	3 (3)
Stage					
IIA	7 (37)	4 (36)	25 (45)	29 (43)	36 (42)
IIB	7 (37)	4 (36)	23 (41)	27 (40)	34 (40)
IIIA	4 (21)	3 (27)	6 (11)	9 (13)	13 (15)
IIIB	1 (5)	0 (0)	2 (3)	2 (3)	3 (3)
Histology					
Invasive ductal carcinoma					
Papillotubular carcinoma	3 (16)	0 (0)	4 (7)	4 (6)	7 (8)
Solid tubular carcinoma	4 (21)	6 (55)	11 (20)	17 (25)	21 (24)
Scirrhous carcinoma	11 (58)	5 (45)	40 (71)	45 (67)	56 (65)
Others	1 (5)	0 (0)	1 (2)	1 (1)	2 (2)
Nuclear grade					
1	10 (53)	1 (9)	34 (61)	35 (52)	45 (52)
2	5 (26)	3 (27)	11 (20)	14 (21)	19 (22)
3	4 (21)	7 (64)	10 (18)	17 (25)	21 (24)
Undetermined	0 (0)	0 (0)	1 (2)	1 (1)	1 (1)
Estrogen receptor					
Positive	12 (63)	2 (18)	47 (84)	49 (73)	61 (71)
Negative	7 (37)	9 (82)	9 (16)	18 (27)	25 (29)
Progesterone receptor					
Positive	10 (53)	0 (0)	30 (54)	30 (45)	40 (47)
Negative	9 (47)	11 (100)	26 (46)	37 (55)	46 (53)
HER2					
Positive (IHC 3+ or FISH+)	6 (32)	4 (36)	8 (14)	12 (18)	18 (21)
Negative	13 (68)	7 (64)	48 (86)	55 (82)	68 (79)
Intrinsic subtype					
Luminal A	10 (53)	2 (18)	43 (77)	45 (67)	55 (64)
Luminal B	3 (16)	0 (0)	4 (7)	4 (6)	7 (8)
HER2 subtype	3 (16)	4 (36)	4 (7)	8 (12)	11 (13)
Triple negative	3 (16)	5 (45)	5 (9)	10 (15)	13 (15)

FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor type 2; IHC, immunohistochemistry.

Discussion

The current study failed to validate the sensitivity of testing using the expression of five genes. However, we became aware of the importance of deciding how to incorporate a new biomarker into clinical practice. Evidence levels of a biomarker are commonly derived from retrospective studies,[18,19] which harbor strong bias due to differing backgrounds. A large cohort or meta-analysis is mandatory to establish usefulness. Prospective trials to evaluate biomarkers have rarely been reported. Simon and simon *et al.* have proposed a refined guideline system for biomarker studies.[20,21] The guideline indicates that level 1 evidence may permit reproducible positive results from high-quality retrospective studies using archived specimens in the prospective trials addressing therapeutic questions, but not biomarkers. However, a prospective trial that would directly address biomarkers is still the gold standard to achieve level 1 evidence. Designing randomized trials for biomarkers presents several challenges.[21] One involves the therapeutic question of accommodation of biomarkers, such as the Tailor X trial of the 21-gene classifiers. The other involves the biomarker question, such as microarray testing of the 70-gene classifier. However,

Table 3. Response and clinical outcome

Sensitivity testing	A	B1	B2	Subtotal of patients in B (B1 + B2) (n = 67)	All patients (n = 86)
	Not performed	Performed			
		Sensitive to paclitaxel	Insensitive to paclitaxel		
Treatment	Paclitaxel (n = 19)	Paclitaxel (n = 11)	FEC100 (n = 56)		
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Pathological response Grade 2 + Grade 3	4 (21.1)	4 (36.4)	7 (12.5)	11 (16.4)	15 (17.4)
Pathologically free metastasis in resected lymph nodes	7 (36.8)	3 (27)	26 (46.4)	29 (43.2)	36 (41.8)
Mean no. pathological positive nodes	3.9	3.8	2.2	2.5	2.8
Pathological disappearance of axillary nodes in pathological responders (grade 2 + 3)	3 (75)	1 (25)	5 (71)	6 (55)	9 (60)
Clinical response (RECIST) CR + PR	10 (53)	6 (55)	30 (54)	36 (54)	46 (54)
Breast conservation	6 (32)	4 (36)	29 (52)	33 (49)	39 (45)

RECIST, response evaluation criteria in solid tumors.

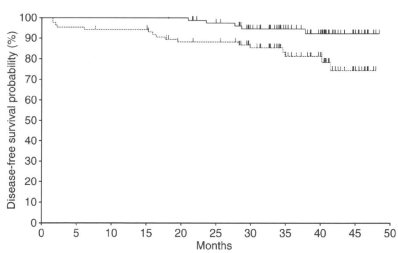

Fig. 2. Kaplan–Meier plot for disease-free survival (dashed line) and overall survival (solid line) in all randomized patients (n = 86).

these trials require a large number of patients to arrive at a definitive conclusion. It is difficult to conduct such a large trial for all possible biomarkers. A relatively smaller number of patients, approximately 100 like in this study, could be reasonable for evaluation in biomarker study design.

The current study failed to enrich responsive patients to treatment with paclitaxel. We expected that prediction of a pathological response would be more than 80% of sensitivity in the new testing. Unexpectedly, the pathological response of the experimental arm was as low as 36.4%. Since the pathological response rate, 21.1%, of the control arm was also lower than 30% as expected, performing the interim analysis in this study took a long time. We decided to terminate the study because we considered that the enrichment of response by sensitivity testing should be minimally more than 50% for clinically meaningful usage or further evaluation by a randomized large phase III study. We did not plan to address the specificity of gene testing, because the specificity could not be yielded from the data of arm B (B1 + B2). The reason was that patients in arm B2, who were judged as insensitive to paclitaxel, did not receive paclitaxel from an ethical point of view. However, we were able to examine the specificity of gene testing in arm A. In arm A (n = 19), 18 patients could be evaluated by gene testing, because of one sampling that contained no cancer cells. Twelve out of 15 patients who failed to obtain a pathological response exhibited as insensitive to paclitaxel by the gene testing. Therefore, the specificity resulted in 80% (12/15). One out of three patients who achieved a pathological response were revealed as sensitive to paclitaxel by the testing. The sensitivity of arm A resulted in 33.3% (1/3), which was similar to that of arm B1 (36.4%, 4/11). The present study aimed to examine whether the gene testing improved sensitivity, but not specificity. The number was too small to obtain a definitive result of specificity. The current study failed to show an enhanced response rate. However, if we conducted a phase II study with a single arm, we would not have been able to obtain such a clear conclusion as early as we did. Therefore, a small randomized study appears to be a robust tool in obtaining a rapid conclusion to evaluate the usefulness of biomarkers.

The methodology of this randomized trial might need further discussion. We wanted to determine whether the selection of patients by new testing could be useful. Thus, we considered that the selection by itself should be randomized. Namely, we compared the outcome for patients who were selected by testing with that of patients who were not selected. This is different from randomized trials that compare a new treatment with a standard therapy. Unbalanced randomization at a 1:4 ratio would minimize the number of patients in control arm A who were not selected by testing. Patients who wished to receive extensive, maximal primary chemotherapy did not enter this trial. One patient withdrew from this trial during her primary chemotherapy because she wanted to receive additional primary chemotherapy. Patients who wished to receive minimal chemotherapy were likely to participate in this trial. Patients with incomplete clearance of axillary tumors could receive additional chemotherapy after surgery. This ethical issue was discussed and approved by institutional review board.

In the current study, the clinical response rate and the conservation rate of the breast were 55% and 36%, respectively, with 27% of patients free from pathological metastasis in resected

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Table 4. Adverse events following preoperative chemotherapy

	Paclitaxel (n = 29)			FEC100 (n = 56)		
	Any grade	Grade 3	Grade 4	Any grade	Grade 3	Grade 4
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Anorexia	6 (21)	0	0	25 (45)	1 (2)	0
Fatigue	22 (76)	1 (3)	1 (3)	44 (79)	2 (4)	0
Nausea	7 (24)	0	0	36 (64)	0	0
Vomiting	2 (7)	0	0	23 (41)	4 (7)	0
Diarrhea	11 (38)	0	0	13 (23)	3 (5)	0
Constipation	15 (52)	0	0	31 (55)	1 (2)	0
Mucositis	0 (0)	0	0	13 (23)	1 (2)	0
Dysgeusia	2 (7)	0	0	4 (7)	0	0
Peripheral neuropathy	26 (90)	1 (3)	0	16 (29)	0	0
Alopecia	29 (100)	NA	NA	56 (100)	NA	NA
Hand-foot syndrome	0 (0)	0	0	2 (4)	0	0
Rash	3 (10)	0	0	1 (2)	0	0
Allergic reaction	1 (3)	0	0	0	0	0
Itching	1 (3)	0	0	0	0	0
Phlebitis	0 (0)	0	0	1 (2)	0	0
Myalgia	2 (7)	0	0	0	0	0
Infection	2 (7)	0	0	13 (23)	5 (9)	2 (4)
Febrile neutropenia	0 (0)	0	0	7 (13)	2 (4)	0
Leukopenia	19 (65)	1 (3)	0	53 (95)	28 (50)	15 (27)
Neutropenia	14 (48)	3 (10)	0	53 (95)	2 (4)	49 (88)
Anemia	15 (52)	0	0	37 (66)	2 (4)	0
Thrombocytopenia	0 (0)	0	0	3 (5)	1 (2)	0
AST elevation	15 (52)	0	0	23 (41)	0	0
ALT elevation	13 (45)	1 (3)	0	22 (39)	0	0
Total bilirubin elevation	3 (10)	0	0	3 (5)	0	0
Creatinine elevation	1 (3)	0	0	1 (2)	0	0
Hyperglycemia	3 (10)	0	0	14 (25)	0	0
All events	212 (27.0)	7 (0.9)	1 (0.1)	494 (32)	52 (3.6)	66 (4.5)

ALT, alanine transaminase; AST, aspartate transaminase; NA, not applicable.

lymph nodes. These results were not satisfactory. However, the study did not aim to improve breast conservation and clearance of axillary metastasis, but rather aimed to minimize exposure to cytotoxic chemotherapy for those sensitive to chemotherapy. Patients with axillary nodes involved were treated by adding adjuvant alternative chemotherapy with FEC100 or docetaxel. The results of disease-free survival and overall survival (81.2% and 94.6% at 3 years) were not assessable for further analysis. The safety profile of paclitaxel or FEC100 was similar to previous reports.[3–5] Both treatments were manageable.

In conclusion, the current study failed to validate sensitivity testing using five-gene expression for primary chemotherapy with paclitaxel in patients with breast cancer. However, a small prospective randomized study is useful for reaching a rapid conclusion on the usefulness of biomarkers. We consider that the present trial design is a prospective randomized phase II trial directly addressing the predictive biomarker question. The current compact trial could be a hallmark to proceed to further large clinical phase III trials.

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Disclosure Statement

The authors have no conflict of interest.

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Cell Injury, Repair, Aging and Apoptosis

Role of Insulin-Like Growth Factor Binding Protein 2 in Lung Adenocarcinoma

IGF-Independent Antiapoptotic Effect Via Caspase-3

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Insulin-like growth factor (IGF) signaling plays a pivotal role in cell proliferation and mitogenesis. Secreted IGF-binding proteins (IGFBPs) are important modulators of IGF bioavailability; however, their intracellular functions remain elusive. We sought to assess the antiapoptotic properties of intracellular IGFBP-2 in lung adenocarcinomas. IGFBP-2 overexpression resulted in a decrease in procaspase-3 expression; however, it did not influence the phosphorylation status of either IGF receptor or its downstream targets, including Akt and extracellular signal-regulated kinase. Apoptosis induced by camptothecin was significantly inhibited by IGFBP-2 overexpression in NCI-H522 cells. Conversely, selective knockdown of IGFBP-2 using small-interfering RNA resulted in an increase in procaspase-3 expression and sensitization to camptothecin-induced apoptosis in NCI-H522 cells. LY294002, an inhibitor of phosphatidylinositol 3-kinase, caused a decrease in IGFBP-2 levels and enhanced apoptosis in combination with camptothecin. Immunohistochemistry demonstrated that intracellular IGFBP-2 was highly expressed in lung adenocarcinomas compared with normal epithelium. Intracellular IGFBP-2 and procaspase-3 were expressed in a mutually exclusive manner. These findings suggest that intracellular IGFBP-2 regulates caspase-3 expression and contributes to the inhibitory effect on apoptosis independent of IGF. IGFBP-2, therefore, may offer a novel therapeutic target and serve as an antiapoptotic biomarker for lung adenocarcinoma. ***(Am J Pathol 2010, 176:1756–1766; DOI: 10.2353/ajpath.2010.090500)***

Insulin-like growth factor-I and -II (IGF-I and -II) are important regulators of cellular metabolism, growth, and survival. When IGFs bind to their receptors, the type I and type II IGF receptors (IGF-IR or IGF-IIR), they activate the downstream signaling cascades via the phosphorylation of tyrosine kinase. Activated IGF-1R transmits signals to the major distinct pathways mitogen-activated protein kinase and phosphatidyl inositol 3-kinase (PI3K), signaling pathways that are highly implicated in the development and progression of neoplasia. IGF's bioavailability is regulated by six high affinity IGF binding proteins (IGFBPs). Secreted IGFBPs by cancer cells interfere primarily with IGF-I or -II through the formation of IGF-IGFBPs complex, which in turn exert an inhibitory effect on IGF-mediated biological functions.

IGF-independent functions of extracellular IGFBPs have long been discussed. Secreted and membrane-associated IGFBP-2 directly binds to proteoglycans and integrins,[1–5] demonstrating IGFBP-2 as a negative or positive regulator of cell adhesion, migration, and invasion in an IGF-independent manner. In the same way, IGFBP-2 positively or negatively regulates cell growth

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and survival in certain types of cancers *in vitro*.[2,6–11] In *in vivo* studies, the growth of mice colorectal adenomas induced by chemical carcinogen was inhibited when they were crossed with IGFBP-2 transgenic mice[12]; however, in contrast, IGFBP-2 exerts oncogenic effects in brain-specific transgenic mice.[13] Thus, increased IGFBP-2 confers advantage or disadvantage for tumor growth, depending on cell type and physiological conditions.[2,14]

Despite these two opposite effects of IGFBP-2 on biological behaviors of cancers, biochemistry and molecular pathology have demonstrated that IGFBP-2 is overexpressed in a wide variety of human malignancies, including glioma,[15] prostate cancer,[16] lung cancer,[17–19] colorectal cancer,[20] ovarian cancer,[21] adrenocortical tumor,[22] breast cancer,[23] and leukemia.[24] Importantly, IGFBP-2 is frequently overexpressed in advanced cancers and is suggested to be involved in the metastatic process.[25] Several potential mechanisms of cancer progression mediated by secreted IGFBP-2 are discussed,[14] but little study has been conducted to the analysis of intracellular-IGFBP-2 functions.

Our aim for this study is to examine the effect of intracellular IGFBP-2 on apoptosis in lung cancer cells and elucidate its molecular mechanism. We also examine the significance of intracellular IGFBP-2 and procaspase-3 in clinical samples and explore the therapeutic implications.

Materials and Methods

Cell Culture and Clinical Samples

The human lung adenocarcinoma cell lines A549, NCI-H460, NCI-H23, NCI-H522, HOP62, COR-L105, and PC14 were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 media supplemented with 10% fetal bovine serum (both medium and serum were from Gibco-BRL, Tokyo, Japan) and 1% penicillin/streptomycin in an atmosphere of 5% CO_2 at 37°C, as previously described.[26]

We also analyzed the mRNA and protein expression in 24 pairs of primary lung adenocarcinomas and corresponding normal lung tissues. All experiments were performed by using a protocol approved by the Institutional Review Board of the Japanese Foundation for Cancer Research (number 2007-1058).

Transient and Stable Transfections

IGFBP-2 cDNA expression construct in pcDNA3.1/Neo (Invitrogen, Carlsbad, CA) was a generous gift from Dr. Hiroaki Kataoka (Section of Oncopathology and Regenerative Biology, Department of Pathology, University of Miyazaki, Japan).[27] Cells were plated at 7×10^5 per well in 60-mm dishes and transfected in triplicate by using the FuGENE 6 Transfection Reagent according to the manufacturer's protocol (Roche Diagnostics, Inc., Indianapolis, IN). We established stable cell lines COR-L105, NCI-H522, and HOP62 overexpressing IGFBP-2 after 4 weeks of selection in 400 μg/ml of neomycin.

RNA Preparation and Real-Time RT-PCR

The cells and frozen tissue were collected for RNA extraction by using an RNeasy Kit (Qiagen, Valencia, CA), and total RNA was applied for first-strand cDNA synthesis with a high capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Gene-specific probes and primer were obtained from Universal Probe-Library (number 25, Roche Applied Science, Tokyo, Japan), and primer sequences were as follows: 5′-TTGCA-GACAATGGCGATGACC-3′ (IGFBP-2 forward); 5′-GGG-ATGTGCAGGGAGTAGAGG-3′ (IGFBP-2 reverse). PCR was performed in 96-well plates by using the LightCycler 480 System (Roche Applied Science). All reactions were performed at least in triplicate. The relative amounts of all mRNAs were calculated by using the comparative threshold cycle (CT) method after normalization to human β2 microglobulin.

Cell Lysis and Immunoblotting

To obtain total protein lysates, frozen tissue and cells were homogenized and dissolved in radioimmunoprecipitation assay buffer (150 mmol/L of NaCl, 1.0% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L of Tris, pH 7.6) containing proteinase inhibitors and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan). The protein concentration of each lysate was determined by using a protein assay reagent kit (BioRad, Hercules, CA). The total cell lysate was applied on 4% to 12% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred electrophoretically from the gel to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked for 1 hour in blocking buffer (5% low-fat dried milk in Tris-buffered saline) and probed with the primary antibodies overnight. After being washed, the protein content was made visible with horseradish-peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Piscataway, NJ). Signal densities were quantitatively determined by ImageJ 1.36 b software (NIH, Bethesda, MD). The primary antibodies used were raised against IGFBP-2 (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), caspase-3, phosphorylated (Tyr1135/1136) and total IGF-1R β, phosphorylated (Ser 473) and total Akt, phosphorylated (Thr 202/Tyr 204) and total Erk1/2, cleaved poly ADP-ribose polymerase (PARP; all obtained from Cell Signaling Technology, Danvers, MA), and β-actin (Sigma, St. Louis, MO). LY294002 was purchased from Sigma.

Caspase Activity Assay

Caspase activities were measured by using the Caspase-Glo 3/7 assay kit according to the manufacturer's instruction (Promega, Madison, WI). Cells (5×10^3 cells/well) were placed in a 96-well culture plate, followed by treatment with dimethyl sulfoxide (DMSO) vehicle or 200 nmol/L of camptothecin for 24 hours. One hundred microliters of Caspase-Glo 3/7 reagent was added to each well and incubated for 1 hour at room temperature.

The culture media with the reagent served as blank, and blank control value was subtracted from each sample value. Luminescence of all samples was measured by using a Tecan Spectrafluor Plus (Wako, Osaka, Japan).

Enzyme-Linked Immunosorbent Assay

IGFBP-2 concentrations in media of cell culture were determined with IGFBP-2 Duoset enzyme-linked immunosorbent assay (ELISA) Development system (R and D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, capture antibody was plated in a 96-well microplate and incubated overnight at room temperature. One hundred microliters of supernatant of culture media or IGFBP-2 standard were added into plate and incubate for 2 hours at room temperature, followed by the immunoreaction with IGFBP-2 detection antibody. IGFBP-2 concentration was calculated from the standard curve. All experiments were performed in duplicate or triplicate.

RNA Interference

Small-interfering RNA (siRNA) oligonucleotides for IGFBP-2 (Santa Cruz Biotechnology) and a negative control (Invitrogen) were transfected into the cells. Transfection was performed by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol of siRNA and 10 μl of Lipofectamine RNAiMAX were mixed in 1 ml of Opti-MEM medium (10 nmol/L of final siRNA concentration). After 20 minutes of incubation, the mixture was added to the suspended cells and these were plated on dishes. Cells were harvested at 24-hour intervals until 72 hours after transfection.

Cell Proliferation and Apoptosis

Cell proliferation was measured as the number of viable cells, as evaluated at 450 nm optical density by using Cell Count reagent SF (Nacalai Tesque). Apoptotic cells were determined by Hoechst 33342 staining, and the apoptosis rate (percent of total population) was evaluated by counting apoptotic and nonapoptotic cells in at least three randomly selected fields.

Immunohistochemistry

Tissue microarrays were constructed from 169 paraffin-embedded lung adenocarcinomas. Briefly, H&E-stained sections containing representative tumor regions were selected. Tissues were punched from cancer areas of each donor block by using tissue cylinders with a diameter of 2 mm and then brought into a recipient paraffin block. Three tumor cores were taken per patient.

Immunohistochemistry was performed on 5-μm thick, formalin-fixed, paraffin-embedded sections by using primary antibodies for IGFBP-2 (C-18, Santa Cruz Biotechnology) and procaspase-3 (Cell Signaling Technology). Antigen retrieval was performed for 30 minutes in citrate buffer for each antibody. The slides were developed by using the labeled streptavidin biotinylated peroxidase method (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. 3,3′-Diaminobenzidine tetrahydro-chloride was used as the chromogen, and hematoxylin was used as the counterstain. A549 xenografts in nude mice were previously established[26] and were used as a positive control. The primary antibody was omitted for negative controls. All immunohistochemical staining was accomplished with a Dako Autostainer (DakoCytomation, Carpenteria, CA) under the same conditions. The staining intensity of IGFBP-2 and procaspase-3 was scored semiquantitatively: positive in less than 25% of cancer cells (weak), positive in 25% to 50% of cancer cells (moderate), and positive in more than 50% of cancer cells (strong). Representative score of each patient was defined as the highest score across three cores.

Statistical Analysis

For *in vitro* experiments, statistical analysis was performed by using Welch's *t*-tests. Comparisons of IGFBP-2 mRNA levels in clinical samples were made by using paired *t*-test analysis. Dose/time dependency of drugs was determined by the confidence interval (CI) based test of slope of the linear regression. Concentrations of drugs that suppressed cell proliferation to 50% of levels exhibited by control cells (IC50) were derived from the dose-response curve. Correlation between IGFBP-2 and caspase-3 expression in immunohistochemistry was evaluated by performing the Fisher's exact test. For all analyses, $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed by using the statistical programming language of R (http://www.R-project.org; accessed February 1, 2010) and Statistika (Statsoft, Inc., Tulsa, OK).

Results

IGFBP-2 Is Expressed and Secreted in Lung Adenocarcinoma Cell Lines

At first, intracellular IGFBP-2 expression levels were examined in various lung cancer cell lines by the use of Western blot. IGFBP-2 was highly expressed in A549, NCI-H460 cells, but expressed at very low levels in HOP62 and COR-L105 cells (Figure 1A).

The levels of secreted IGFBP-2 in media were measured by ELISA. Secreted IGFBP-2 levels correlated with intracellular protein levels obtained by Western blot (Figure 1B).

IGFBP-2 Expression Is Regulated Transcriptionally and Posttranslationally

IGFBP-2 expression is physiologically up-regulated by the energy restriction or insulin-dependent diabetes mellitus.[28,29] To determine whether the supplement of nutrients can alter IGFBP-2 expression in lung cancer cells, we examined the effects of glucose or serum depletion on IGFBP-2 expression in A549 cells. Glucose depletion significantly reduced IGFBP-2 levels at both protein and mRNA levels ($P = 0.0017$), whereas serum depletion did not ($P = 0.311$; Figure 2A). IGFBP-2 protein and mRNA levels were dependent on glucose concentration (Figure 2B). These findings suggest that IGFBP-2 expression in

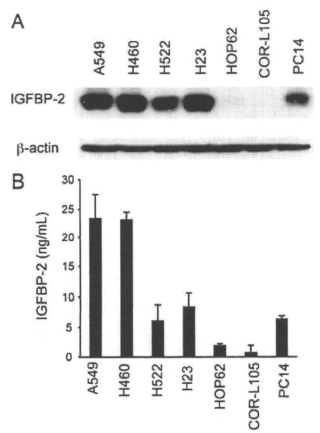

Figure 1. A: Basal levels of intracellular IGFBP-2 protein in seven lung adenocarcinoma cell lines. Cells (5×10^5) were plated in a 60-mm dish and cultured for 48 hours. The protein extracts from each cell line were resolved by SDS-polyacrylamide gel electrophoresis and blotted with an antibody against IGFBP-2. β-actin served as internal control. A representative data from two independent experiments is shown. **B:** Conditioned media containing a different amount of IGFBP-2 in lung adenocarcinoma cell lines. Secreted IGFBP-2 was measured, under the same conditions as above, by ELISA. Values represent means ± SD.

cancer cells is glucose-dependent and is regulated by a mechanism that is distinct from normal cells.

It has been reported that IGFBP-2 expression is regulated by the PI3K-PTEN (phosphatase and tensin homolog deleted on chromosome 10) pathway in prostate and glioblastoma cells.[30] Thus, extracellular and intracellular IGFBP-2 levels were evaluated in lung cancer cells treated with LY294002, a PI3K inhibitor. PTEN protein was detected in all cell lines, except PC14, as described previously.[26] Secretion of IGFBP-2 protein was suppressed in all cell lines by the treatment of LY294002 to varying degrees (Figure 2C). The effect of LY294002 on IGFBP-2 expression showed a significant dose dependence ($P = 0.0048$) and time course dependence (95% CI: 0.134 to 0.18, control; 0.029 to 0.043, LY294002) in A549 cells (Figure 2, D and E). Intracellular IGFBP-2 levels were also decreased with LY294002 (Figure 2F). Interestingly, a fraction of IGFBP-2 protein was degraded into approximately 20 kDa after treatment with LY294002 (Figure 2F). Conversely, IGFBP-2 mRNA was significantly increased with LY294002 ($P < 0.005$; Figure 2G), suggesting the existence of a compensatory feedback mechanism.

IGFBP-2 Overexpression Suppresses Procaspase-3 Expression and Confers Resistance for Drug-induced Apoptosis

To address whether IGFBP-2 is involved in apoptotic event, IGFBP-2 was enforced in cells with low endogenous IGFBP-2 levels, and then caspase expression was examined. IGFBP-2 overexpression resulted in a remarkable increase in intracellular IGFBP-2 levels in COR-L105, NCI-H522, and HOP62 cells compared with vector control (Figure 3A). Secreted IGFBP-2 levels of these cells were also increased corresponding to the levels of intracellular IGFBP-2 (Figure 3B). Intriguingly, IGFBP-2 overexpression resulted in a substantial decrease in procaspase-3 expression (Figure 3A). However, caspase-9 was not decreased (Figure 3A), suggesting IGFBP-2 specifically inhibits caspase-3 expression. Despite a higher amount of IGFBP-2 secretion into media, no significant changes were found in the IGF signaling pathway including phosphorylation statuses of IGF-1R, Akt, or Erk1/2 (Figure 3A). These findings suggest that IGFBP-2-mediated caspase-3 inhibition occurs in an IGF-independent manner.

Next, to examine whether IGFBP-2 involves in apoptotic event, we compared the sensitivity of IGFBP-2 overexpressing cells and vector control cells to an apoptosis inducer, camptothecin. IGFBP-2 overexpressing and vector control H522 cells were exposed to 20 to 1000 nmol/L of camptothecin for 24 hours, and the cell proliferation and caspase-3 activity were analyzed. The results indicated that IGFBP-2 overexpressing H522 cells were significantly resistant to camptothecin (EV, IC50 = 686 nmol/L; BP-2, IC50 > 1000 nmol/L; Figure 3C). As expected, caspase-3 activity was significantly decreased in IGFBP-2 overexpressing cells compared with vector control cells on treatment with camptothecin ($P < 0.02$; Figure 3D). Apoptosis was evaluated by Hoechst 33342 staining and PARP cleavage. Enforced IGFBP-2 significantly inhibited PARP cleavage, as determined by Western blot (Figure 3E), and reduced camptothecin-induced apoptotic cells in H522 cells ($P = 0.003$; Figure 3F). Similar results were obtained with the treatment of cisplatin or etoposide (data not shown).

IGFBP-2 Inhibition Up-Regulates Procaspase-3 Expression and Promotes Drug-Induced Apoptosis

To further elucidate the effects of IGFBP-2 on caspase-3, gene silencing for IGFBP-2 was performed in A549 and H522 cells. IGFBP-2 knockdown induced an increase in procaspase-3 expression until 72 hours after siRNA treatment in both cell lines (Figure 4A). No significant active form of cleaved caspase-3 was identified (data not shown). As is the results with IGFBP-2 overexpression, no substantial change was found in caspase-9. In addition, IGFBP-2 siRNA also decreased the phosphorylation status of IGF-1R. This effect might be because of a rapid decrease in both intracellular and extracellular IGFBP-2. Although IGFBP-2 knockdown resulted in morphological changes such as shrinkage in A549 cells, no substantial increase in apoptosis was identified by Hoechst 33342 staining or PARP cleavage (data not shown).

We now asked whether IGFBP-2 inhibition sensitizes cells for drug-induced apoptosis. Figure 4B shows the cell proliferation of IGFBP-2 knockdown and negative control cells with a treatment of camptothecin. IGFBP-2