# Increased Akt and phosphorylated Akt expression are associated with malignant biological features of prostate cancer in Japanese men

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OBJECTIVE

To investigate the relationship between the expression of Akt (a serine/threonine kinase that plays a central role in tumorigenesis), phosphorylated Akt (p-Akt), prostate cancer tumour grade, androgen receptor (AR)-staining score, and Ki67 labelling index (LI) in Japanese men.

PATIENTS, MATERIALS AND METHODS

The expression and activation of the cell survival protein Akt was analysed by immunohistochemical staining of paraffin-embedded tissue microarray sections of prostate carcinoma taken from 52 Japanese men who under radical prostatectomy. The correlation between the expression of Akt and p-Akt, and their relationship to primary Gleason grade, AR expression and Ki-67 LI was investigated.

RESULTS

The expression of Akt and p-Akt were positively related to primary Gleason grade (Fisher's exact test, $P = 0.002$ and $P = 0.032$, respectively). Both AR-staining score and Ki67 LI were were positively related to the expression of Akt (both $P < 0.001$) and p-Akt ($P < 0.001$ and $P = 0.008$, respectively). There was a significant positive correlation between the expression of Akt and p-Akt (Spearman's correlation, $r = 0.644$, $P < 0.001$).

CONCLUSIONS

Increased expression of both Akt and p-Akt were associated with higher tumour grade as well as a higher AR-staining score and Ki67 LI. These data indicate that Akt and p-Akt might be molecular markers for detecting malignant biological features of prostate cancer in the Japanese population.

KEYWORDS

prostate cancer, Akt, phosphorylated Akt, androgen receptor

## INTRODUCTION

Prostate cancer is the commonest visceral malignancy and the second leading cause of cancer mortality in men in Western countries [1]. Prostate cancer is initially dependent on androgens for growth. Most tumours are primarily treated with surgery or radiation therapy, but disease that is in an advanced stage at diagnosis and disease that progresses after primary treatment are managed with androgen-ablation therapy (AAT) [2]. Unfortunately, most tumours treated with AAT alone become resistant to AAT [3]. When this occurs, the prognosis is poor because a standard therapy has yet to be established for this group. Thus, there is an urgent need to improve our understanding of the biology of prostate cancer and to establish new prognostic markers to aid in the selection of unfavourable prognostic groups for whom adjuvant therapy is indicated.

In 1997, the tumour-suppressor gene, PTEN was identified as a gene that is mutated in multiple sporadic tumour types [4,5]. In prostate cancer cell lines (PC3 and LNCaP) a deletion or point mutation of PTEN have been reported [4,5]. PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signalling pathway [6]. This pathway is important for cell proliferation and cell survival, and PTEN antagonizes the activity of the PI3K oncoproteins, as it functions as a tumour suppressor. Thus, the PTEN-PI3K-signalling pathway functions as a crucial regulator of cell survival decisions. Important downstream targets of the PTEN-PI3K signalling pathway include the serine-threonine protein kinase B family, or Akt. Members of this family, Akt-1, Akt-2 and Akt-3, are broadly expressed in most organs and tissues. The relative contribution of specific Akt isoforms to the biological activities regulated by them was unknown until recently, but the development of 'knockout' mice for each of the isoforms, as well as isoform-specific small interfering RNAs for analysis in cultured cells, have revealed the specific functions of individual Akt proteins. Briefly, a targeted disruption of Akt-1 in mice has shown its role in the regulation of body size and adipogenesis [7]. As for Akt-2, disruption of this isoform in mice leads to severe insulin resistance and diabetes [8], and for Akt-3, mice lacking in Akt-3 had in 20%

decrease in brain size [9]. The net result of the activation of all the Akt isoforms is protection from apoptosis and an increase in cell proliferation. Accumulating evidence suggests that the activation of this key survival kinase plays an important role in tumour development. In a transgenic mouse model (murine prostate Akt kinase transgenic, MPAKT) a constitutively active Akt was overexpressed in mouse prostate epithelia, which resulted in prostate intraepithelial neoplasia [10]. Prostate cancer cell line studies have shown that reduced apoptotic response, release from the cell-cycle block, and regulation of androgen receptor (AR) expression can be triggered by overexpression and activation of Akt [11–13]. Immunohistochemical studies of Akt expression in human prostate cancer tissues showed that Akt is upregulated in prostate cancer and that expression was correlated with tumour progression in Western populations [14–17]. Recently, Le Page *et al.* [18] examined the expression and localization of the three Akt isoforms, suggesting a particular role of Akt-1 expression as a prognostic marker depending on its localization (cytoplasmic or nuclear).

Prostate cancer incidence and mortality rates have been rising over the last two decades, but remain lower in Asian countries, including Japan, than in Western countries. The difference might be partly due to genetic factors, such as ras mutations [19] and polymorphism of the AR and enzymes that participate in androgen metabolism, as well as dietary or environmental factors [20]. Hence, investigating the risk factors for disease progression in Japanese men with prostate cancer is important for advancing our understanding of the disease. In the present study, prostate carcinoma tissue taken from Japanese men at RP was analysed for the expression of Akt and phosphorylated Akt (p-Akt), and their relationships with tumour grade, AR-staining score, Ki67 labelling index (LI), and PSA-defined disease-free survival rate were evaluated.

## PATIENTS, MATERIALS AND METHODS

Prostate tissue specimens were obtained from 52 consenting patients who had radical prostatectomy (RP) for prostate cancer between June 1997 and August 2001 at Kyoto University Hospital, Japan. None of the patients had a diagnosis of metastatic cancer or had received hormonal therapy before RP.

| Variable | Median (range) or N |
|---|---|
| Age, years | 68 (50–76) |
| PSA level before RP, ng/ml | 9.9 (3.8–120) |
| Follow-up, months | 81.6 (19.2–106.9) |
| Pathological T stage | |
| pT2 | 15 |
| pT3 | 37 |
| Gleason sum | |
| 5 | 2 |
| 6 | 2 |
| 7 | 45 |
| 8 | 0 |
| 9 | 3 |
| 10 | 0 |
| Primary Gleason score | |
| 3 | 39 |
| 4 | 12 |
| 5 | 1 |
| Surgical margin | |
| negative | 17 |
| positive | 35 |

*TABLE 1*
*The demographic and clinicopathological features of the 52 patients*

All clinical and pathological data were obtained from medical records and are summarized in Table 1. The date of PSA failure was considered to be when the first blood sample after RP yielded a detectable PSA level (≥0.1 mg/mL). When serum PSA did not decline to <0.1 ng/mL after RP, the date of failure was defined as the date of RP. The patients were followed until 31 March 2006, for a median (range) of 81.6 (19.2 to 106.9) months. The tissue microarrays (TMAs) were constructed as previously described [21]. Three cores from tumour-bearing tissue were obtained from each patient, giving a total of 156 cancer spots.

Standard indirect immunoperoxidase procedures were used on the paraffin-embedded sections. Anti-AKT1/2 polyclonal antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antip-Akt (Ser473) monoclonal antibody (Cell Signalling Technology, Beverly, MA, USA), anti-AR monoclonal antibody (2F12 Novocastra, Newcastle, UK), and anti-Ki67 monoclonal antibody (MIB-1, DAKO, Kyoto, Japan) were used as primary antibodies at dilutions of 1 : 400, 1 : 100, 1 : 100, and 1 : 100, respectively. The chromogen was 3,3′-diaminobenzidine tetrahydrochloride. Tumours with known positivity were used as positive controls for all antibodies. For the negative controls, primary antibodies were omitted.

The results of Akt and p-Akt staining were interpretation by two of the authors (T.I. and Y.S.) 'blinded' to the clinicopathological data and all spots in the arrays were examined. For Akt and p-Akt, total staining was scored as the product of the staining intensity (on a scale of 0–3) and the percentage of cells stained, resulting in a scale of 0–300 [16]. Staining intensity in the cytoplasm and plasma membrane was defined as follows: no cells stained positively, 0; weak staining, 1; moderate staining, 2; and strong staining, 3; as previously described [16]. Nuclear staining was considered representative of Ki67, while both cytoplasmic and nuclear staining of the epithelium was considered positive for AR. The Ki67 LI was defined as the percentage of the nuclear area stained. The AR-staining score was defined as follows: weak AR staining, 1; moderate , 2; and strong, 3; as previously described [21]. To evaluate the prognostic value of each marker, the mean staining score of Akt, p-Akt and AR (the sum of the score of the three cancer spots in each case divided by three) and the mean Ki67 LI (the sum of the LIs of the three cancer spots in each case divided by three) were calculated for each case. Expression of Akt, p-Akt, the Ki67 LI, and AR-staining score were dichotomized according to a threshold of 140, 140, 5, and 1.6, respectively.

Spearman's rank correlation coefficient was used to analyse the correlation between Akt

*FIG. 1. Representative immunohistochemical staining of Akt, p-Akt, AR, and Ki67 expression in human prostatic adenocarcinoma. (A, C, E and G, Gleason grade 4; B, D, F and H, Gleason grade 3). (A) Immunohistochemical staining with an Akt antibody showing strong cytoplasmic staining; this would be given a score of 270 [90% of the cells × 3 (strong staining)]. (B) Immunohistochemical staining with an Akt antibody showing no staining; this would be given a score of 0. (C) Immunohistochemical staining with a p-Akt antibody showing strong cytoplasmic staining; this would be given a score of 285 [95% of the cells × 3 (strong staining)]. (D) Immunohistochemical staining with a p-Akt antibody showing no staining; this would be given a score of 0. (E) Immunohistochemical staining with an AR antibody showing strong cytoplasmic and nuclear staining; this would be given a score of 3. (F) Immunohistochemical staining with an AR antibody showing no staining; this would be given a score of 0. (G) Immunohistochemical staining with a Ki67 antibody showing strong nuclear staining; the Ki67 LI was 12. (H) Immunohistochemical staining with a Ki67 antibody showing no staining; the Ki67 LI was 0.*

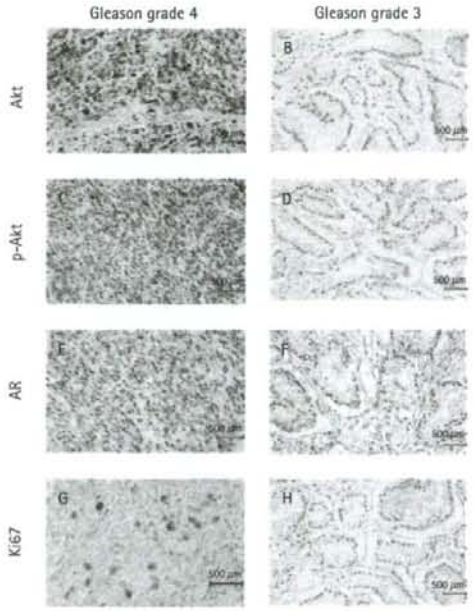
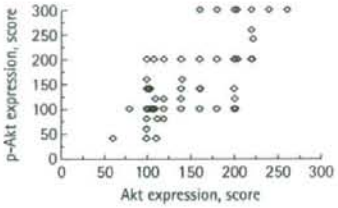



*FIG. 2. Scatterplot of Akt and p-Akt expression scores in each cancer spot. Akt expression correlates with that of p-Akt. Spearman's correlation, $r = 0.644$; $P < 0.001$.*




and p-Akt staining scores in each of the 156 cancer spots. The chi-squared test or Fisher's exact probability test were used to analyse the relationship between the staining score of Akt or p-Akt and the AR-staining score, the Ki67 LI or primary Gleason grade in each cancer spot. Cox proportional-hazards model was used for univariate and multivariate survival analyses to evaluate the risk factors associated with PSA failure. To obtain a multivariate model with the statistically significant variables, a stepwise-selection procedure was used. Categorical variables included age, preoperative PSA level, stage, Gleason sum, primary Gleason score, TMA Gleason sum, TMA primary Gleason score, surgical margin, the Ki67 LI and the staining score of Akt, p-Akt and AR. A $P < 0.05$ was considered to indicate statistical significance.

## RESULTS

Of the 156 cancer spots investigated, 122 had a primary Gleason grade of <4 and the remaining 34 were ≥4. Akt expression was localized mainly in the cytoplasm (Fig. 1A). In rare cases, Akt was localized in the nuclei. The expression of p-Akt was similar (Fig. 1C). AR expression was located in both the cytoplasm and nuclei (Fig. 1E). Ki67 expression was localized in the nuclei (Fig. 1G). The median (range; interquartile range, IQR) Akt and p-Akt staining scores were 120 (60–300; IQR 100–200) and 100 (0–300; IQR 100–200), respectively. There was a significant positive correlation between p-Akt and Akt expression levels (Spearman's correlation, $r = 0.644$, $P < 0.001$; Fig. 2). The median (range) AR-staining score was 1 (1–3; IQR 1–2) and Ki67 LI was 2.85 (0–49.7; IQR 1.15–8.00).

The staining intensity of Akt, p-Akt, AR, and Ki67 in each cancer spot was classified as low or high. In tumours with a primary Gleason grade of <4, 58.2% of spots had a low staining score for Akt and 62.3% had a low staining score for p-Akt. By contrast, in tumours with a primary Gleason grade of ≥4, 73.5% of spots had a high staining score for Akt and 58.8% had a high staining score for p-Akt. There was a highly significant difference in the staining scores for Akt and p-Akt between tumours with a primary Gleason grade of <4 and tumours with a primary Gleason grade ≥4 (Fisher's exact test, $P = 0.002$ and $P = 0.032$, respectively; Table 2).

In all, 79 spots (50.6%) had low AR expression and the remaining 77 (49.4%) had high AR expression. In the spots with low AR expression, 75.9% had low Akt expression and 74.7% had low p-Akt expression. In the spots with high AR expression, 74.0% had high Akt expression and 59.7% had high p-Akt expression. There was a highly significant difference in the staining score for Akt and p-Akt between tumours with low AR expression and tumours with high AR expression (both $P < 0.001$, Table 2).There was also a strong relationship between the Ki67 LI and the expression of Akt and p-Akt ($P < 0.001$ and $P = 0.001$, respectively; Table 2).

The 5-year PSA-defined disease-free survival rate for the 52 patients was 59.6% (31

*TABLE 2 Expression of Akt and p-Akt in relation to primary Gleason grade, AR expression and Ki-67 LI in each cancer spot (156 spots)*

| Variable, n (%) | Akt expression (score) | | | p-Akt expression (score) | | |
|---|---|---|---|---|---|---|
| | low intensity* | high intensity† | P‡ | low intensity* | high intensity† | P‡ |
| Primary Gleason grade | | | | | | |
| low grade (<4) | 71 (58.2) | 51 (41.8) | 0.002 | 76 (62.3) | 46 (37.7) | 0.032 |
| high grade (≥4) | 9 (26.5) | 25 (73.5) | | 14 (41.2) | 20 (58.8) | |
| AR expression (score) | | | | | | |
| low intensity (<1.6) | 60 (75.9) | 19 (24.1) | <0.001 | 59 (74.7) | 20 (25.3) | <0.001 |
| high intensity (≥1.6) | 20 (26.0) | 57 (74.0) | | 31 (40.3) | 46 (59.7) | |
| Ki-67 LI (%) | | | | | | |
| low intensity (<5) | 69 (71.1) | 28 (28.9) | <0.001 | 64 (66.0) | 33 (34.0) | 0.008 |
| high intensity (≥5) | 11 (18.6) | 48 (81.4) | | 26 (44.1) | 33 (55.9) | |

**low intensity score <140; †high intensity score ≥ 140; ‡Fisher's exact test.*

patients). Univariate analysis showed that Akt, p-Akt, AR expression, Ki67 LI, pT stage, primary Gleason score, surgical margin, TMA Gleason sum and TMA primary Gleason score were significant factors for PSA-defined disease-free survival (Table 3). The relative importance of each variable was determined by multivariate Cox proportional-hazards model analysis. Stepwise inclusion of variables in the model showed that the significant factors were primary Gleason score ($P = 0.043$, hazard ratio, HR, 2.59), surgical margin status ($P = 0.006$, HR 8.39) and Ki67 LI ($P < 0.001$, HR 6.58) (Table 3).

*TABLE 3 Univariate and multivariate analysis (Cox regression models) for PSA-defined treatment failure*

| Variable | HR (95% CI) | P |
|---|---|---|
| **Univariate analysis** | | |
| Age (<70 vs ≥70 years) | 0.70 (0.29, 1.66) | 0.414 |
| PSA level (≥10 vs <10 ng/ml) | 2.07 (0.86, 4.99) | 0.107 |
| pT stage (≥T3 vs <T3) | 4.61 (1.07, 20) | 0.040* |
| Gleason sum (≥7 vs <7) | 1.43 (0.9, 2.28) | 0.130 |
| Primary Gleason score (≥4 vs <4) | 3.26 (1.37, 7.8) | 0.008* |
| TMA Gleason sum (≥7 vs <7) | 3.67 (1.08, 12.49) | 0.037* |
| TMA primary Gleason score (≥4 vs <4) | 6.17 (2.07, 18.45) | 0.011* |
| Surgical margin (positive vs negative) | 5.79 (1.34, 24.93) | 0.018* |
| Akt score (≥140 vs <140) | 2.53 (1.02, 6.29) | 0.046* |
| p-Akt score (≥140 vs <140) | 2.54 (1.02, 6.31) | 0.044* |
| AR (high vs low) | 2.45 (0.99, 6.11) | 0.049* |
| Ki67 LI (positive vs negative) | 6.64 (2.40, 18.33) | <0.001* |
| **Multivariate analysis** | | |
| Primary Gleason score (≥4 vs <4) | 2.59 (1.03, 6.53) | 0.043* |
| Surgical margin (positive vs negative ) | 8.39 (1.86, 37.86) | 0.006* |
| Ki67 LI ( positive vs negative) | 6.58 (2.26, 19.15) | <0.001* |

**P < 0.05.*

## DISCUSSION

The present results show the importance of Akt expression and activation as molecular markers for malignant biological features of prostate cancer in the Japanese population. The expression and activation of Akt was increased in high Gleason grade prostate cancers. Liao *et al.* [14] also reported that an increase of Akt expression correlated with Gleason pattern in human prostate cancer, while Le Page *et al.* [18] reported that there was no correlation between Akt-1, Akt-2, or Akt-3 and Gleason score, surgical grade or pathological stage. Expression of p-Akt was reported by Malik *et al.* [15] to be significantly greater in Gleason grades 8–10 vs all other grades, while Ayala *et al.* [17] showed that p-Akt expression was correlated only with clinical staging of tumour, and Le Page *et al.* [18] showed that p-Akt overexpression was correlated only with preoperative PSA level. The reasons for the discrepancies among these results are unknown, but differences in the patient cohorts or in statistical analysis might be contributory.

Fenic *et al.* [22] reported that when comparing prostate intraepithelial neoplasia, primary carcinomas and metastases as ordered groups (with increasing malignancy), both PTEN mRNA ($r = -0.49$, $P = 0.06$) and its protein ($r = -0.36$, $P = 0.09$) showed a progressive decreasing trend. PTEN protein expression was moderate to strong in 27 of 58 primary tumours (46.5%), weak in 24 (41.3%), and negative in eight (13.8%). Of 15 metastases, PTEN protein expression was moderate to strong in four, weak in six, and negative in five [22]. In the present early-stage prostate cancer tissues, ≈49% of cancer spots showed high expression of Akt and there was a significant correlation between the expression of Akt and p-Akt ($r = 0.644$, $P < 0.001$). These results suggest that both the loss of PTEN and the increase in Akt expression might activate Akt in early-stage human prostate cancer and play an important role in the poor differentiation of prostate cancer cells. However, the precise role of this early up-regulation of Akt needs further examination.

In the present study, expression of Akt and p-Akt were strongly related with the Ki67 LI, the cell proliferation antigen. Akt is a well-known regulator of cell survival, promoting cell survival by phosphorylating and inactivating the pro-apoptotic protein BAD [23]. Akt also stimulates cell cycle progression by suppressing AFX-mediated transcription of target genes such as the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [24]. Thus, activation of Akt regulates not only survival but also proliferation of cancer cells. Ghosh *et al.* [25] verified these results *in vitro* using LNCaP cells and its androgen-independent clone C4-2, and *in vivo* using human prostate cancer tissues. C4-2 cells expressed higher Akt activation and increased proliferation than LNCaP cells, similar to cases with poor clinical outcome [25]. They also reported in an immunohistochemical study of 74 human prostate cancer specimens that there was a strong correlation between the expression of p-Akt and the Ki67 LI [25], which is compatible with the present results.

In the present immunohistochemical study, there was a strong relationship between the expression of Akt or p-Akt and AR expression. Manin *et al.* [13] showed that AR protein levels are greatly reduced after treatment of LNCaP cells with the PI3K/Akt pathway inhibitor LY294002. LY294002 does not alter AR mRNA accumulation, suggesting that translational or post-translational mechanisms might be the main factors responsible for decreased AR expression. Consistent with this possibility, it was reported that Akt activates mRNA translation by phosphorylation and inactivation of the translational repressor eIF4E-binding proteins [26]. The mammalian target of rapamycin, which is important in the regulation of protein translation, is a direct target for Akt [27] and might be the mediator of the growth factor's effects on AR protein expression. Recently Xin *et al.* [28] suggested synergy of Akt and AR exists in the progression of prostate cancer. In the present study, there was a significant relationship between Akt/p-Akt expression and AR expression, suggesting that the PI3K/Akt pathway might control AR-mediated progression of prostate cancer. To our knowledge, this is the first immunohistochemical study showing a strong relationship between the expression of Akt or p-Akt and the expression of AR in prostate cancer. Previously published data show that the significance of the AR in prostate cancer after RP remains limited and inconclusive. Li *et al.* [29] reported that patients with higher levels of AR that had a RP had a higher degree of malignancy, more advanced disease progression, and worse biological recurrence-free survival, while another study showed that low AR expression correlated with histopathological grade and indicated a poor prognosis [30]. Although the correlations between the expression of AR and prognosis are not fully understood, considering that the androgen/AR complexes are known to promote tumour cell proliferation and survival [31], it is reasonable to think that hormonally naïve prostate cancer treated with RP might take advantage of higher AR status, which eventually might lead to enhanced AR activity, resulting in more growth advantage under a low androgen environment due to ageing [32]. Moreover, this aggressiveness was confirmed by a significant positive relationship between AR immunostaining and Ki67 LI in our previous study [21] and a study by Li *et al.* [29].

Kreisberg *et al.* [16] reported that in a group with weak p-Akt staining (score 1–100) the PSA failure rate was half, whereas, in a moderate staining group (101–200) the rate was 66.7%, and in a strong staining group (201–300) it was 91.3% . Increased p-Akt was an independent predictor of the probability of PSA failure. Although p-Akt was not an independent prognostic factor according to multivariate analysis in the present study, a similar tendency was observed: in the weak p-Akt staining group the PSA failure rate was 23.5%, whereas in the moderate and strong staining groups the rates were 43.3 and 60.0%, respectively.

In conclusion, immunohistochemical analysis of prostate cancer specimens from Japanese men showed that the aggressive form of prostate cancer is accompanied by increased levels of Akt and p-Akt. The close correlation between the expression of Akt and p-Akt shows that the higher expression of Akt might increase its activity and play an important role in the poor differentiation of prostate cancer cells. Although Akt and p-Akt were not independent prognostic factors according to our multivariate analysis, the expression of Akt and p-Akt were related with tumour grade, AR-staining score and the Ki67 LI, and are therefore thought to be molecular markers for malignant biological features of prostate cancer. We think this is the first report from an Asian country showing a strong relationship between the aggressiveness of prostate cancer and expression of Akt and p-Akt. Despite the previously noted differences in gene alterations between prostate cancers from Japan and Western countries, the present study highlights the similarities of Akt and p-Akt alterations between these populations. One potential implication of the present study is that Akt and AR alterations are more central in prostate-cancer biology, in comparison to the other gene alterations, which might be linked to environmental factors. Further investigation of the Akt pathway might therefore reveal its involvement in the progression of prostate cancer.

## CONFLICT OF INTEREST

None declared.

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Abbreviations: **AAT**, androgen-ablation therapy; **PI3K**, phosphatidylinositol 3-kinase; **AR**, androgen receptor; **p-Akt**, phosphorylated Akt; **LI**, labelling index; **RP**, radical prostatectomy; **TMA**, tissue microarray; **IQR**, interquartile range; **HR**, hazard ratio.

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# Polymorphisms of fibroblast growth factor receptor 4 have association with the development of prostate cancer and benign prostatic hyperplasia and the progression of prostate cancer in a Japanese population

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**Fibroblast growth factor receptor 4 (FGFR4) is a member of a family of transmembrane receptors with ligand-induced tyrosine kinase activity. The Glycine (Gly) to Arginine (Arg) polymorphism at codon 388 (Gly388Arg), which encodes an amino acid in the transmembrane part of the FGFR4 gene, was reported to be associated with an increased risk in some carcinomas. We investigated the association between the Gly388Arg polymorphism or the G or A polymorphism at intron 11 (rs2011077) of FGFR4, which was located 1,213 base pairs apart from the Gly388Arg polymorphism, and the risk of prostate cancer or benign prostate hyperplasia (BPH), and the prostate cancer disease status in Japanese men. Genotypes of Gly388Arg and rs2011077 polymorphisms of FGFR4 were determined in 492 patients with prostate cancer, 165 patients with BPH and 179 male controls. Regarding the Gly388Arg polymorphism, individuals with the ArgArg genotype had a 2.207- and 1.958-fold increased risk of prostate cancer and BPH, and a 1.804-fold increased risk of metastatic prostate cancer compared with those with the GlyGly genotype. Regarding the rs2011077 polymorphism, individuals with the GG genotype had a 6.260- and 3.033-fold increased risk of prostate cancer and BPH, and a 5.550-fold increased risk of metastatic prostate cancer compared with those with the AA genotype. Our results indicate that the FGFR4 Arg allele of the Gly388Arg polymorphism and the G allele of the rs2011077 polymorphism have a significant impact on the development of prostate cancer and BPH, and the progression of prostate cancer in a Japanese population.**


**Key words:** prostate cancer; polymorphism; fibroblast growth factor receptor; benign prostatic hyperplasia

Prostate cancer is the second common cancer among men in most western populations.[1] There are striking differences in the age-adjusted incidence of prostate cancer between different racial groups and between different geographic regions of the world.[2] Rates among African-Americans are the highest in the world (275.8 per 100,000 person-years), followed by Caucasian-Americans (171.9 per 100,000 person-years).[3] The incidence of 19.9 per 100,000 person-years in Japanese men is among the lowest in the world.[4] On the other hand, Japanese immigrants in the United States have experienced a marked increase in prostate cancer incidence, although the rates in Japanese men in Los Angeles and the San Francisco Bay Area are still less than in whites.[5,6] These epidemiological data demonstrate that both genetic and environmental factors play a significant role in the incidence of prostate cancer.

Fibroblast growth factor receptor 4 (FGFR4) is a member of a tyrosine kinase receptor (TKR) family that displays multiple biological activities, including mitogenic and angiogenic activity, with a consequent crucial role in cell differentiation, development, hormonal and proliferative signaling,[7] in response to more than 20 known ligands. A few studies demonstrated that it was expressed in normal human prostate, PIN, prostate cancer, prostate cancer cell lines and the immortalized human prostate epithelial cell line.[8–10] Potent activators of FGFR4 (FGF2, FGF6 and FGF8) and FGFR4 itself are frequently overexpressed in human prostate cancer.[8,11] Recently, Bange *et al.*[12] reported a Gly to Arg polymorphism at codon 388 (Gly388Arg) in the FGFR4 gene (*FGFR4*) and found that the presence of the *FGFR4* Arg388 allele had a substantial negative impact on disease-free survival in patients with breast cancer with lymph node metastasis, although other groups have not observed a similar effect in their patients with breast cancer.[13] Bange *et al.*[12] also reported that the presence of the *FGFR4* Arg388 allele was associated with metastasis and poor prognosis in colon cancer. In head and neck squamous cell carcinoma, an association was demonstrated between the high expression of *FGFR4* Arg388 allele and poor clinical outcome.[14] In prostate cancer, Wang *et al.*[10] demonstrated that the presence of *FGFR4* Arg388 allele was associated with clinical aggressiveness. These findings were supported by independent groups with similar results in soft tissue sarcoma and lung adenocarcinoma,[15,16] although contradictory results have also been presented.[17–19] Benign prostate hyperplasia (BPH) has an inheritable genetic component,[20] and Boget *et al.*[21] reported a close relationship between FGFR and BPH. Therefore, an analysis that includes men with BPH in a control group may mask the role of *FGFR4* polymorphism in prostate cancer. The present study was conducted to explore the association of the Gly388Arg or the *G* to *A* polymorphism in *FGFR4* intron 11 (rs2011077) with the development of prostate cancer and BPH, and the progression of prostate cancer in a Japanese population. In addition, we measured linkage disequilibrium (LD) between two polymorphisms.

## Material and methods

### *Subjects*

A total of 836 subjects, consisting of 492 patients with prostate cancer, 165 patients with BPH and 179 male controls treated at Akita University Medical Center and related community hospitals were enrolled in this study. Medical records were used to obtain clinical data, with approval from the institute's ethics committee and with informed consent. All patients with prostate cancer were pathologically diagnosed from specimens obtained from transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. The clinical or pathological stage of prostate cancer at the time of diagnosis was determined by reviewing the medical records based on the Tumor-Node-Metastasis system. Prostate cancers were classified into Stage A ($T_{1a-b}N_0M_0$), Stage B ($T_{1c-2}N_0M_0$), Stage C ($T_{3-4}N_0M_0$) and Stage D ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$) by the modified Whitmore–Jewett system. In patients who underwent radical prostatectomy, the final pathological stage was applied; and in patients without radical prostatectomy, the

*Abbreviations:* FGFR, fibroblast growth factor receptor; TKR, tyrosine kinase receptor; BPH, benign prostate hyperplasia; LD, linkage disequilibrium; LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; OR, odds ratios; CI, confidence intervals; aOR, age-adjusted OR.

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clinical stage was applied. Pathological grading of prostate cancer was determined according to the General Rules for Clinical and Pathological Studies on prostate cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is based on the WHO criteria and according to the Gleason score. All pathological grading was based on needle biopsy specimens in patients of Stages B–D and surgical specimens in patients of Stage A. Well-, moderately and poorly differentiated carcinoma generally corresponded to Gleason scores of 2–4, 5–7 and 8–10, respectively. In the present study, because the two grading systems were used by local pathologists, the tumor grade system was newly categorized as follows: (*i*) low-grade cancer including well-differentiated or Gleason 2–4 carcinomas; (*ii*) intermediate-grade cancer including moderately differentiated or Gleason 5–7 carcinomas; and (*iii*) high-grade cancer including poorly differentiated or Gleason 8–10 carcinomas. All patients with BPH had various lower urinary tract symptoms of different degrees and enlarged prostates measured with transabdominal ultrasound. The serum PSA levels of all patients with BPH were measured by the Tandem-R assay (Hybritech, San Diego, CA). Patients who had elevated serum PSA levels (4.0 ng/ml or more) had transrectal sextant biopsies to eliminate the possibility of prostate cancer. The 179 male controls were over 60 years old with no voiding symptoms, selected from admitted patients in nonurological departments in community hospitals. They all had serum PSA levels less than 4.0 ng/ml and showed no signs of prostate cancer or prostatic enlargement by digital transrectal ultrasound. Serum PSA was measured using the Tandem-R assay in most cases. When serum PSA was measured by kits other than the Tandem-R assay, the measured PSA level was adjusted to that of the Tandem-R assay using a formula published elsewhere.[22]

*Genotyping of FGFR4 polymorphisms*

We analyzed 2 single nucleotide polymorphisms of *FGFR4*, Gly388Arg and rs2011077. The 2 polymorphisms are located 1,213 base pairs apart on the chromosome. DNA was extracted from blood samples collected from each patient and control using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or by the standard method with proteinase K digestion followed by phenol/chloroform extraction. Polymerase chain reaction (PCR) amplification of fragments encompassing polymorphic sites was performed in a 15-μl PCR reaction mix containing ~20 ng genomic DNA, 1× PCR buffer supplied by the manufacturer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$, 50 pmol of each primer and 1.0 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). For the Gly388Arg polymorphism, after the 10-min step of initial denaturation at 94°C, PCR was carried out at 94°C for 1 min, at 64°C for 1 min and at 72°C for 1 min for a total of 35 cycles, followed by a 10-min final extension step at 72°C in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, Waltham, MA). The 168-bp production containing the *BstN* I polymorphic site was PCR-amplified using forward primer 5'-GACCGCGACGACGCCCG-GAGCCGA-3' and reverse primer 5'-AGAGGGAAGCGGGA-GAGCTTCTGC-3'. For the rs2011077 polymorphism, the reaction situation was the same as that of Gly388Arg except that the annealing temperature was 55°C. The 102-bp production containing the *Bfa* I polymorphic site was PCR-amplified with forward primer 5'-AGAGAGGTAGAGGGCCTGTGGAGCTGACTA-3' and reverse primer 5'-GAAGAATTTGGTGTGACAGGCTTG-3'. After confirmation of successful PCR amplification by loading 5 μl of the reaction products on 2.5% agarose gel electrophoresis, each PCR product was digested overnight with 5 U of restriction enzyme *BstN* I (60°C) or *Bfa* I (37°C) (New England Biolabs, Beverly, MA). For the Gly388Arg polymorphism, restriction fragments were 109-, 37- and 22-bp for the Gly allele and 82-, 37-, 27- and 22-bp for the Arg allele. For the rs2011077 polymorphism, restriction fragments were 72- and 30-bp for the *G* allele and 102-bp for the *A* allele. Restriction enzyme-treated PCR products were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining for analyses of *FGFR4* polymorphisms. To avoid genotyping errors caused by DNA contamination, incomplete digestion or other technical failures, we repeated the experiment at least twice for all samples.

*Statistical analyses*

All data were entered into an access database and analyzed using Excel 2003 (Microsoft, Richmond, WA and Washington, DC) and SPSS, version 15.0J (SPSS, Chicago, IL) software. The mean age of the subjects in the three groups was examined using the independent *t* test. Hardy–Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using the Pearson $\chi^2$ test. Case–control data (allelic frequencies and genotype distributions) were analyzed using the Pearson $\chi^2$ test. A multivariate logistic regression model was used to assess the association between the disease risk and genotypes by calculating odds ratios (OR) and 95% confidence intervals (CI) adjusted by age as a confounding factor. We hypothesized the Arg allele of the Gly388Arg polymorphism as an inherent genetic risk factor for prostate cancer, BPH and prostate cancer progression. Statistical modeling was performed on the relative risk of the ArgArg or GlyArg genotype against the GlyGly genotype for Gly388Arg independently using the logistic regression model adjusted by age. In addition, the gene dosage effect of the Arg risk allele was assessed by modeling the linear effect on the log odds scale for each risk allele in multivariate logistic regression, such as the genotypes ArgArg, GlyArg and GlyGly, which were valued as "2," "1" and "0," respectively. To rs2011077, the *G* allele was hypothesized as the inherent genetic risk factor for prostate cancer, BPH and prostate cancer progression. We also performed statistical modeling analysis for the *G* allele as mentioned above. The disease-free interval was defined as the period from the date of radical retropubic prostatectomy to the date when PSA was more than 0.4 ng/mL. Cancer-specific death was defined as death from prostate cancer or from other causes strongly associated with the progression of prostate cancer. Survival time was calculated from the date of prostate cancer diagnosis to the day of death due to cancer-specific death, with deaths from other causes censored. The relationship between the polymorphisms and the disease-free survival in prostate cancer patients of Stages A–D1 or cancer-specific survival in patients with prostate cancer of Stage D2 was estimated by the Kaplan–Meier method and evaluated by the log-rank test. LD was measured between two polymorphisms in 3 groups. All statistical tests and *p* values were 2-tailed and results were considered significant at $p < 0.05$.

## Results

*Subject characteristics*

The present study included 492 cases of pathologically confirmed prostate cancer, 165 cases of BPH with lower urinary tract symptoms and 179 male controls. The mean age ± SD was 70.34 ± 7.43, 70.53 ± 9.41 and 70.98 ± 7.38 years, respectively. No significant difference in the mean age was found between patients with prostate cancer and controls ($p = 0.322$) or between patients with BPH and controls ($p = 0.621$).

*Genotype and allelic frequencies of FGFR4 Gly388Arg and rs2011077 polymorphisms*

The genotype distributions of *FGFR4* Gly388Arg and rs2011077 polymorphisms are presented in Table I. The distribution of genotypes for the two polymorphisms in the control group (for Gly388Arg, $\chi^2 = 0.147$, $df = 2$, $p = 0.701$; for rs2011077, $\chi^2 = 3.168$, $df = 2$, $p = 0.075$) were consistent with Hardy–Weinberg equilibrium. Statistical analyses of genotype frequency for the polymorphisms showed a significant difference between the prostate cancer group and the control group (Gly388Arg: $p = 0.002$, rs2011077: $p < 0.001$), and between the BPH group and the control group (Gly388Arg: $p = 0.023$, rs2011077: $p = 0.009$). The allelic frequencies of *FGFR4* Gly388Arg and rs2011077

TABLE I – ALLELIC FREQUENCIES AND GENOTYPE DISTRIBUTIONS (%) OF FGFR4 POLYMORPHISMS IN PROSTATE CANCER PATIENTS, BPH PATIENTS AND MALE CONTROLS

| | No. | Gly388Arg FGFR4 | | | | | rs2011077 FGFR4 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Allele (Freq) | | Genotype (%) | | | Allele (Freq) | | Genotype (%) | | |
| | | *Arg* | *Gly* | *ArgArg* | *ArgGly* | *GlyGly* | *A* | *G* | *AA* | *GA* | *GG* |
| Prostate cancer group | 492 | 462 (0.47) | 522 (0.53) | 133 (27.0) | 196 (39.8) | 163 (33.1) | 511 (0.52) | 473 (0.48) | 113 (23.0) | 285 (57.9) | 94 (19.1) |
| BPH group | 165 | 149 (0.45) | 181 (0.55) | 42 (25.5) | 65 (39.4) | 58 (35.2) | 197 (0.60) | 133 (0.40) | 53 (32.1) | 91 (55.2) | 21 (12.7) |
| Control group | 179 | 137 (0.38) | 221 (0.62) | 25 (14.0) | 87 (48.6) | 67 (37.4) | 251 (0.70) | 107 (0.30) | 83 (46.6) | 85 (47.5) | 11 (6.1) |
| Tumor stage | | | | | | | | | | | |
| A | 10 | 7 (0.35) | 13 (0.65) | 1 (10.0) | 5 (50.0) | 4 (40.0) | 11 (0.55) | 9 (0.45) | 3 (30.0) | 5 (50.0) | 2 (20.0) |
| B | 245 | 224 (0.46) | 266 (0.54) | 57 (23.3) | 110 (44.9) | 78 (31.8) | 288 (0.59) | 202 (0.41) | 63 (25.7) | 162 (66.1) | 20 (8.2) |
| C | 70 | 55 (0.39) | 85 (0.61) | 15 (21.4) | 25 (35.7) | 30 (42.9) | 77 (0.55) | 63 (0.45) | 22 (31.4) | 33 (47.1) | 15 (21.4) |
| D1 | 25 | 28 (0.56) | 22 (0.44) | 10 (40.0) | 8 (32.0) | 7 (28.0) | 14 (0.28) | 36 (0.72) | 3 (12.0) | 8 (32.0) | 14 (56.0) |
| D2 | 142 | 148 (0.52) | 136 (0.48) | 50 (35.2) | 48 (33.8) | 44 (31.0) | 121 (0.43) | 163 (0.57) | 22 (15.5) | 77 (54.2) | 43 (30.3) |
| Tumor grade | | | | | | | | | | | |
| Low | 15 | 14 (0.47) | 16 (0.53) | 4 (26.7) | 6 (40.0) | 5 (33.3) | 21 (0.70) | 9 (0.30) | 6 (40.0) | 9 (60.0) | 0 (0.0) |
| Intermediate | 213 | 193 (0.45) | 233 (0.55) | 48 (22.5) | 97 (45.5) | 68 (31.9) | 225 (0.54) | 195 (0.46) | 49 (23.0) | 131 (61.5) | 33 (15.5) |
| High | 197 | 189 (0.48) | 205 (0.52) | 60 (30.5) | 69 (35.0) | 68 (34.5) | 195 (0.49) | 199 (0.51) | 43 (21.8) | 109 (55.3) | 45 (22.8) |

polymorphisms are presented in Table I. As for the Gly388Arg polymorphism, there was a significant difference in allelic frequency between patients with prostate cancer and controls ($p = 0.005$, Table I), but no significant difference between patients with BPH and controls ($p = 0.067$, Table I). Regarding the rs2011077 polymorphism, there was a significant difference in allelic frequency between patients with prostate cancer and controls ($p < 0.001$, Table I), and between patients with BPH and controls ($p = 0.004$, Table I).

### *FGFR4 Gly388Arg or rs2011077 genotypes and the risk of prostate cancer or BPH*

To evaluate the risk of prostate cancer and BPH according to the *FGFR4* genotypes, logistic regression analysis was conducted with adjustment for age at the time of diagnosis. For the Gly388Arg polymorphism, a significant increased risk of prostate cancer or BPH was found in men with the ArgArg genotype (for prostate cancer: age-adjusted OR [aOR] = 2.207, 95% CI = 1.320–3.690, $p = 0.003$; for BPH: aOR = 1.958, 95% CI = 1.065–3.597, $p = 0.030$) compared with the GlyGly genotype (Table II). For the rs2011077 polymorphism, a significant increased risk of prostate cancer was found in men with the *GG* genotype (aOR = 6.260, 95% CI = 3.152–12.433, $p < 0.001$) or the *GA* genotype (aOR = 2.497, 95% CI = 1.717–3.630, $p < 0.001$) compared with the *AA* genotype. When *GG*, *GA* and *AA* genotypes were valued as 2, 1 and 0, respectively, the presence of the *G* allele was shown to increase the risk of prostate cancer with a gene dosage effect (aOR = 2.500, 95% CI = 1.871–3.339, $p < 0.001$). As for BPH, a significantly increased risk was found in the *GG* genotype (aOR = 3.033, 95% CI = 1.352–6.807, $p = 0.007$) and the *GA* genotype (aOR = 1.700, 95% CI = 1.077–2.683, $p = 0.023$) compared with the *AA* genotype. When *GG*, *GA* and *AA* genotypes were valued as 2, 1 and 0, respectively, the presence of the *G* allele was shown to increase the risk of BPH with a gene dosage effect (aOR = 1.724, 95% CI = 1.218–2.441, $p = 0.002$).

### *Genotypes of FGFR4 Gly388Arg or rs2011077 polymorphisms and disease status of prostate cancer*

We examined the relationship between FGFR4 Gly388Arg or rs2011077 polymorphisms and the prostate tumor stage or grade at the time of diagnosis (Table II). Regarding the tumor stage and the Gly388Arg polymorphism, patients with prostate cancer with the ArgArg genotype had a 1.804-fold increased risk of metastatic prostate cancer ($p = 0.015$) compared with the GlyGly genotype. For the rs2011077 polymorphism, patients with prostate cancer with the *GG* genotype had a 5.550-fold increased risk of metastatic prostate cancer ($p < 0.001$) compared with the *AA* genotype. There was no statistically significant result between Gly388Arg or rs2011077 polymorphisms and the prostate cancer grade.

### *Genotypes of FGFR4 Gly388Arg or rs2011077 polymorphisms and prognosis of prostate cancer*

Of the 492 patients with prostate cancer, cancer-specific survival and disease-free survival data were available in 117 patients of Stage D2 and in 141 patients of Stages A–D1, respectively. Regarding cancer-specific survival from prostate cancer and the two polymorphisms, there was no statistical significance among the different genotype groups of the Gly388Arg ($p = 0.313$) (Fig. 1) or rs2011077 ($p = 0.852$) polymorphisms. There was also no statistical significance regarding disease-free survival and the Gly388Arg ($p = 0.840$) (Fig. 2) or rs2011077 ($p = 0.971$) polymorphism.

### *Linkage disequilibrium between Gly388Arg and rs2011077 polymorphisms*

The *D'* values of the group that had patients with prostate cancer, the group that had patient with BPH and controls were 0.471, 0.657 and 0.849, respectively.

TABLE II – STATISTICAL ANALYSES RESULTS OF THE GENOTYPE FREQUENCY

| | Gly388Arg polymorphism aOR (95% CI, $p$) | | | rs2011077 polymorphism aOR (95% CI, $p$) | | |
|---|---|---|---|---|---|---|
| | GlyGly | GlyArg | ArgArg | AA | GA | GG |
| Study group | | | | | | |
| Prostate cancer *versus* control | 1 | 0.939 (0.641–1.375, 0.747) | 2.207 (1.320–3.690, 0.003) | 1 | 2.497 (1.717–3.630, <0.001) | 6.260 (3.152–12.433, <0.001) |
| BPH *versus* control | 1 | 0.871 (0.540–1.404, 0.570) | 1.958 (1.065–3.597, 0.030) | 1 | 1.700 (1.077–2.683, 0.023) | 3.033 (1.352–6.807, 0.007) |
| Tumor stage | | | | | | |
| Stage D *versus* Stage A + B + C | 1 | 0.867 (0.550–1.366, 0.539) | 1.804 (1.120–2.906, 0.015) | 1 | 1.492 (0.894–2.491, 0.126) | 5.550 (3.016–10.214, <0.001) |
| Tumor grade | | | | | | |
| High *versus* Low + Intermediate | 1 | 0.722 (0.460–1.132, 0.156) | 1.237 (0.753–2.035, 0.401) | 1 | 1.003 (0.626–1.608, 0.989) | 1.757 (0.963–1.016, 0.066) |

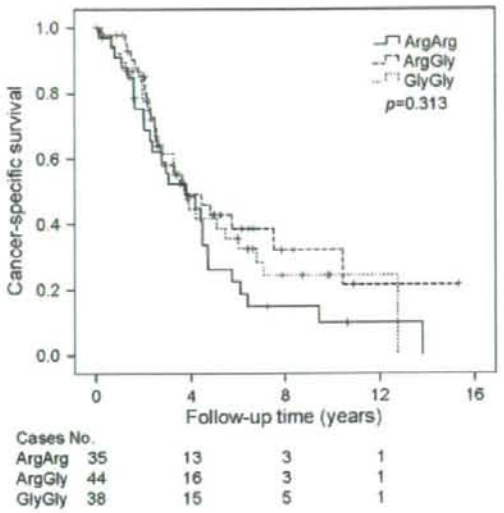


FIGURE 1 – Genotypes of Gly388Arg and the cancer-specific survival of Stage D2 prostate cancer.

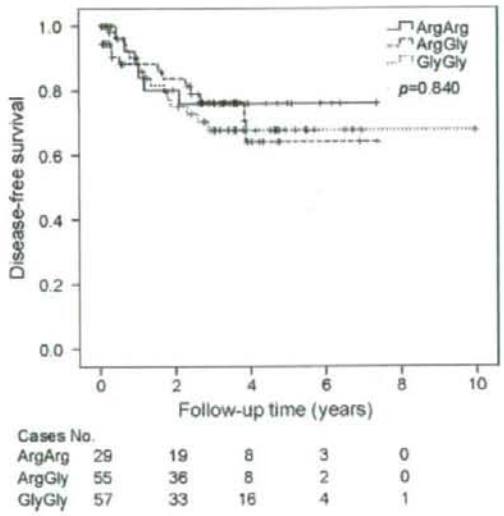


FIGURE 2 – Genotypes of Gly388Arg and the disease-free survival of Stage A–D1 prostate cancer.

## Discussion

The present study showed a significant association between the Arg allele of the *FGFR4* Gly388Arg polymorphism and the development and progression of prostate cancer in a native Japanese male population. Our results further indicate that the Arg allele had a recessive effect on the development and progression of prostate cancer (Table II). These results are in line with a study on prostate cancer in whites and African-Americans by Wang *et al.*[10] However, it is noticeable that although the risk of prostate cancer

in the Japanese population was the lowest among the 3 races, the Arg388 allele frequency in the Japanese population was the highest among patients with prostate cancer from the 3 races, whereas the African-Americans had a contradictory result (0.47 in the Japanese population in our study, 0.36 in whites and 0.12 in African-Americans in Wang *et al.*'s study[10]). This suggests that other environmental or genetic factors must influence the effects of the FGFR4 Arg388 allele.

Although there is no direct biological relationship between prostate cancer and BPH, it is well conceivable that genetic polymorphisms affecting levels of growth factors, including FGFR4, may have a significant impact on both prostate cancer and BPH, because both disease conditions are under the influence of common growth factors. Normal growth patterns in the prostate may result not only from increased cell proliferation but also from decreased levels in programmed cell death.[23] Any imbalance between the physiological process of cell proliferation and cell death may lead to changes in prostate size with the subsequent development of abnormalities in the gland. In the present study, patients with BPH were excluded from the control group to eliminate the confounding effect of the *FGFR4* polymorphism, and we found that men with the ArgArg genotype have a 1.958-fold increased risk of developing BPH ($p = 0.030$) compared with men with the GlyGly genotype. In addition, Bange *et al.*[12] reported that the Arg388 allele was associated with progression in patients with colon cancer. Stadler *et al.*[24] found that FGFR4 Gly388 could reduce a breast cancer cell line response to lysophosphatidic acid (LPA) by downregulating the LPA receptor Edg-2. LPA plays an important role in activation of the mitogenic extracellular signal-regulated kinase (ERK) pathway and in regulating mitogenic signaling and the growth of prostate cancer cells.[25] More recently, Sahadevan *et al.*[11] reported that FGFR4 may be an important target to disrupt FGF signaling in prostate cancer. Taken together, it suggested that FGFR4 Arg388 could enhance the development of prostate cancer and BPH by upregulating LPA signaling, which promotes mitosis mediated by ERK. On the other hand, Wang *et al.*[26] found that Ehm2 expression was upregulated in prostate cancer cell lines and prostate cancer tissues, and the expression of the Arg388 of FGFR4 resulted in increased Ehm2 expression, which may lead to decreased adhesion to Collagen IV; so, Arg388 may be associated with the metastatic phenotype in cancers.[27] Furthermore, Wang *et al.*[10] reported that the expression of *FGFR4* Arg388 in immortalized prostate epithelial cells resulted in increased cell motility and invasion and upregulation of the urokinase-type plasminogen activator receptor, which is known to promote invasion and metastasis.[28] These may explain the relationship between the Arg388 of FGFR4 and the increased metastatic disease status of prostate cancer observed in the present study.

The Gly388Arg polymorphism lies in the transmembrane domain of FGFR4. Mutations in the transmembrane domains of TKRs have been implicated in the induction of pathological phenotypes. These mutations are believed to stabilize TKR dimers, and thus promote unregulated signaling. For example, achondroplasia is often attributed to a Gly388Arg mutation in the transmembrane domain of FGFR3.[29] To further explore the association of the polymorphism of the transmembrane domain of FGFR4 and the risk of prostate cancer and BPH, we searched for common polymorphisms around the Gly388Arg polymorphism in the NCBI SNP database literature. As a result, the rs2011077 polymorphism in intron 11 was found, which had 0.471 frequency and 1,213 base distance to the Gly388Arg polymorphism. We found that the *G* allele was more frequently found in patients with prostate cancer and in patients with BPH, and it might increase the risk of prostate cancer and BPH with a gene dosage effect (Table II). The present data suggested that the Gly388Arg polymorphism and rs2011077 polymorphism were in strong linkage disequilibrium and tightly linked in controls; however, they were not in tight linkage in the group that had patients with prostate cancer. This was in line with the results that the *G* allele of the rs2011077 polymorphism had a gene dosage effect on the development of prostate cancer and BPH, yet the Arg allele seemed to have a recessive influence. Although the rs2011077 polymorphism is located in the intron, it remains unknown whether it may have any significant biological effect on FGFR4 protein or the *FGFR4* expression level. In addition, the polymorphism might be in strong linkage disequilibrium with other unknown polymorphisms, which have a significant biological influence on prostate cancer and BPH development.

Although our study showed that the Arg388 allele and the *G* allele of the rs2011077 polymorphism of FGFR4 was associated with the advanced status of prostate cancer, no relationship was observed between polymorphisms of FGFR4 and the prognosis of prostate cancer, which seemed contrary to the results of several studies that the Arg388 allele of FGFR4 was associated with the prognosis of various cancers.[10,12,14–16] This might be due to the influence of other factors on the prognosis of prostate cancer, one of which is the complexity of the FGFR4 signaling pathway. Studies on the role of FGFR4 in carcinogenesis provide evidence for the complexity of the FGF/FGFR signaling pathway in different tumor types.[30,31] This complexity was reflected to some degree by the contradictory results of the relationship between the Gly388Arg polymorphism and the prognosis of several cancers,[15,16,18,19] and even the same cancers, although other factors might be involved. For localized prostate cancer, clinicopathologic characteristics, such as the Gleason score and the status of the surgical margin, may mask the relationship between polymorphisms of FGFR4 and the prognosis of prostate cancer. In addition, most patients with prostate cancer of Stage D2 and some patients with prostate cancer of Stage A–D1 in our study received androgen deprivation therapies (ADT). It has been demonstrated that genetic polymorphisms can influence tumor response and the severity of adverse effects of chemotherapy.[32–34] Our recent data showed that IGF-I and CYP19 polymorphisms had a significant impact on the prognosis of patients of Stage D2, suggesting that some genetic factors are related to the progression of bone metastasis, hormone-independent growth of tumor cells or the response to ADT.[35] Therefore, other polymorphisms may mask the relationship between polymorphisms of FGFR4 and the prognosis of Stage D2 prostate cancer. All of these factors may cloud the cancer prognosis.

In conclusion, the present results indicate that the Arg allele of Gly388Arg polymorphism and the G allele of rs2011077 polymorphism in the transmembrane domain of *FGFR4* have a significant impact on the development of prostate cancer and BPH, and the progression of prostate cancer in Japanese men. Further studies with extensive haplotype analyses are warranted to delineate the significance of each haplotype of the *FGFR4* locus containing the 2 polymorphisms in more Japanese subjects and other racial-ethnic groups.

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The Prostate 68:321–335 (2008)

# Candidate Genes Involved in Enhanced Growth of Human Prostate Cancer Under High Fat Feeding Identified by Microarray Analysis

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**BACKGROUND.** Several studies have suggested that a high fat diet (HFD) may be a risk factor of prostate cancer (PCa). As a first step to delineate the molecular mechanisms underlying the enhanced progression of PCa under HFD, we investigated the differential gene expressions of a human PCa xenograft under HFD and a low fat diet (LFD).

**METHODS.** LNCaP cells were subcutaneously injected in 20 nude mice, which were equally divided into two groups, the HFD group and LFD group. Oligonucleotide microarray analyses were performed using mice xenografts from HFD and LFD, and the results of candidate genes with a significant differential expression were validated by quantitative RT-PCR experiments. As for insulin-like growth factor I receptor (IGF-IR), protein expression levels were further examined by immunohistochemistry in xenograft tissues and in 78 radical prostatectomy specimens.

**RESULTS.** Tumor volume and serum PSA levels were significantly higher in the HFD group than in the LFD group ($P < 0.001$ and $P = 0.006$, respectively). We found 64 up-regulated genes (0.19%) and 14 down-regulated genes (0.04%) with more than twofold differences in the HFD xenograft. *IGF-IR*, *TNFRSF*, and *LPL* showed striking differences in the quantitative RT-PCR experiment. Immunostaining further revealed marked enhanced IGF-IR expression in the HFD xenograft. In human PCa, the lowest IGF-IR immunoreactivity group tended to have the lowest body mass index in both normal and PCa epithelium.

**CONCLUSION.** HFD induced remarkable up- and down-regulation of mRNA of a substantial number of genes. Furthermore, the IGF-I system may be involved in the HFD-associated enhanced progression of PCa. *Prostate 68: 321–335, 2008.* © 2008 Wiley-Liss, Inc.

*KEY WORDS:* **prostate cancer; high fat diet; microarray; insulin-like growth factor I receptor**

## INTRODUCTION

Prostate cancer (PCa) is the most common malignancy and the second leading cause of cancer mortality in the United States. According to estimates of the American Cancer Society, for the year 2005, over 232,090 new cases of PCa were diagnosed, and 30,350 men died of PCa [1]. While the rate of latent PCa is similar between the United States and Japan, the

Abbreviations: PCa, prostate cancer; HFD, high fat diet; LFD, low fat diet; IGF-IR, insulin-like growth factor I receptor; BMI, body mass index.

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incidence of clinically detected PCa is lower in Asia, including Japan [2,3]. Interestingly, the incidence of PCa in Chinese and Japanese men has been reported to increase substantially after migration to the United States [4], and the morbidity and mortality of PCa have been remarkably increasing in Japan for the last few decades [5]. These epidemiological findings including geographic and ethnic differences suggest that lifestyle and/or environmental factors have a substantial influence on the development and progression of PCa [6].

Among the environmental factors, a fatty diet has been suspected to play an important role in the development of PCa [7]. Several epidemiological studies revealed the association between a high fat diet (HFD) and the progression of PCa. According to the review by Fair et al., [8] it was reported that 11 of 14 case-control studies and 4 of 5 cohort studies showed a positive association between increased fat intake and the incidence of PCa. In addition, studies in animal models also showed increased tumor growth with a high fat intake and the inhibition of growth under a low fat intake [9,10]. It has been hypothesized that androgen metabolism, insulin-like growth factor I (IGF-I), hormonal metabolism, and free radicals are involved in the growth-promoting effect of HFD in human PCa [8,11]. In particular, the link between the promoting effect of HFD and the IGF-I axis has been suggested by a few groups [12]. Recently, the presence of diet-gene interaction was suggested and the influence of diet on PCa risk may be modulated by the genetic variation of genes relevant to PCa development [11]; however, the precise molecular mechanisms behind these observed associations are largely unknown.

In the present study, we investigated the effect of HFD on growth and mRNA gene expression profiles by microarray analyses in a PCa tumor xenograft model to investigate the effect of gene-diet interaction on the development of PCa. In addition, genes having a substantial difference in mRNA expression levels were validated.

## MATERIALS AND METHODS

### Cell and Reagents

The LNCaP cell line, which is an androgen-receptor-positive and androgen-sensitive human prostate adenocarcinoma cell line that produces prostate-specific protein such as PSA [13], was obtained from the American Type Cell Culture Collection (Manassas, VA). LNCaP cells were grown in RPMI Medium 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GEMINI Bioproducts, Woodland, CA) and Penicillin-Streptomycin-Fungizone 100 × Concentrated (Cambrex Bio Science, Walkersville, MD) in an atmosphere of 5% $CO_2$ and air at 37°C.

### Animals

Twenty male athymic mice of the BALB/c-nu/nu strain, aged 6–8 weeks, were obtained from Japan SLC (Shizuoka, Japan), and housed, five animals per cage, in a pathogen-free environment. The Institutional Review Board of the Akita University School of Medicine approved all animal experiments.

### Diets

The feeding receptacles were on top of the cages to control food intake, and new food was given without opening the cages twice a week. The diets were prepared and sterilized by CLEA-Japan (Tokyo, Japan). The HFD contained 56.7% calories from fat; the low fat diet (LFD) contained 10.2% calories from fat (Table I). The HFD was stored in a freezing room (−80°C), and LFD in a cold room (4°C).

### Design of Animal Experiments

LNCaP cells were harvested from subconfluent cultures after 1-min exposure to a solution of trypsin-EDTA (Invitrogen, Grand Island, NY). After suspension in RPMI Medium 1640 supplemented with 10% FBS, the cells were counted with the aid of a Coulter Counter, and cell viability was confirmed by trypan blue dye exclusion. LNCaP cells were finally resuspended in ice-cold BD Matrigel (BD Bioscience, Bedford, MA) at a final concentration of $1 \times 10^6$ cells/0.25 ml Matrigel and 0.25 ml RPMI medium. Each

**TABLE I. Ingredients of Experimental Diets**

| | Low fat diet (%) | High fat diet (%) |
|---|---|---|
| Casein | 17.5 | 24.5 |
| Albumen | 3.6 | 5.0 |
| L-cystine | 0.3 | 0.4 |
| Beef tallow | 2.0 | 15.9 |
| Safflower oil[a] | 2.5 | 20.0 |
| Cellulose | 4.0 | 5.5 |
| Maltodextrin | 2.0 | 8.3 |
| Lactose | 2.3 | 6.9 |
| Sucrose | 10.0 | 6.8 |
| Cornstarch | 51.1 | — |
| AIN93 vitamin mix | 1.0 | 1.4 |
| AIN93G mineral mix | 3.5 | 5.0 |
| Choline bitartrate | 0.3 | 0.4 |
| Tert-butyl hydroquinone | 0.0 | 0.0 |
| Fat Kcal | 10.2 | 56.7 |
| Energy (Kcal) | 365.5 | 507.6 |

[a]Two experimental diets containing different concentration of Safflower oil as a source of fat were used. Oleic acid is rich in the Safflower oil used this experiment.

mouse was given a subcutaneous inoculation of $10^6$ tumor cells in the hindlimb with a disposable syringe equipped with a 26-gauge needle.

All animals were started on a 10.2% LFD, beginning at the time of tumor cell inoculation. At the end of 4 weeks, when measurable tumors had formed in all mice, the mice were assigned to receive each diet. Tumor dimensions were recorded weekly, and tumor volumes were calculated using the formula: length (cm) × weight (cm) × height (cm) × 0.5236 [14]. Fifteen weeks after tumor cell inoculation, blood samples were collected via the orbital sinus after anesthesia, and all mice in each group were euthanized. Tumor tissues were obtained at the termination of the experiments. Serums were stored at −70°C until analyzed. Half of tumor tissues were snap-frozen in liquid nitrogen, and other halves were fixed in formalin and then embedded in paraffin blocks for immunohistochemistry.

Human serum PSAs were measured by ELISA (SRL, Tokyo, Japan). Tumor volumes versus time from tumor cell inoculation for each animal at the change of diet were evaluated at the termination period.

### RNA Extraction

For microarray analyses, total RNA was extracted from one tumor tissue in the HFD group with enhanced growth and one in the LFD group with typical slow growth. For semiquantitative and quantitative RT-PCR analyses, total RNA was extracted from tumor tissues from several mice distinct from that used in microarray experiment to validate the results of the microarray analyses. Total RNAs were isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA), and purified using the RNAsay system (Qiagen, Hilden, Germany). The quality of RNAs was checked in 1 M Tris–HCl (pH 7.5) using an Agilent 2100 bioanalyzer (Agilent Technology, Palo Alto, CA).

### Microarray Analysis

We entrusted the microarray analyses to Filgen, Inc. (Aichi, Japan). Two independent analyses were performed using different batches of oligonucleotide microarray. The human 35-K oligonucleotide arrays utilized herein consisted of 70-mer oligonucleotide probes (manufactured by Operon, Huntsville, AL), representing 34,580 genes. Prehybridization of the microarray was performed for 1 hr at 42°C in a solution containing 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin (BSA). The microarray was then washed at room temperature in distilled water, and dried by centrifugation at 200*g* for 5 min.

Labeling of the aRNA was performed from 2 μg of each extracted total RNA using RNA Transcript SureLABEL Core Kit (Takara, Japan) and Cy5-UTP (Amersham Bioscience, NJ). The 3 μg labeling aRNA was added to a solution containing 0.04 M Tris-acetate, 0.1 M potassium acetic acid, and 0.03 M magnesium acetic acid, and heated for 10 min at 94°C. Hybridization for 16–20 hr at 42°C was performed in 80 μl solution containing 5 × SSC, 0.1% SDS, 10% formamide, and heat-denatured labeling aRNA. After hybridization, the microarray was washed at room temperature with 2 × SSC containing 0.1% SDS for 4 min, once with 0.1 × SSC for 4 min, and three times with 0.1 × SSC for 1 min, and dried by centrifugation. The fluorescence images of Cy5 dye channels were obtained using a GenePix 4000B scanner (Axon Instruments, CA). Fluorescent hybridization signals of the microarray slide were analyzed with Array-Pro Analyzer ver 4.5 software (Media Cybernetics, Silver Spring, MD). The signal intensity of each spot and its local background were quantified, and net intensity was calculated by taking the background to the raw intensity. The database was analyzed using Microsoft Excel. The genes were categorized using the Microarray Data Analysis Tool ver 1.0 (supplied by Filgen, Inc.), based on the Gene-Ontology database http://geneontology.org/.

### Semiquantitative RT-PCR and Primers

One microgram of total RNA was reverse transcribed using oligo $(dT)_{20}$ primer and SuperScript™ III RT (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. PCR cycling conditions were as follows: one cycle of 94°C for 10 min, followed by 22–34 cycles (primer dependant) of 94°C for 15 sec, 54 to 60°C (primer dependant) for 30 sec, 74°C for 60 sec, then 72°C for 5 min. Fifteen microliters of RT-PCR reaction mixture contained 1.5 μl 10 × PCR buffer, 1.5 μl dNTP mix, 1.2 μl $MgCl_2$, 0.6 μl each forward and reverse primer, 8.6 μl $ddH_2O$, and 0.6 U Ampli-Taq Gold DNA polymerase (PE Applied Biosystems, Branchburg, NJ). RT-PCR primers for selected genes are listed in Table II. Amplified PCR products were visualized on a 2% agarose gel. Beta-actin was used as the internal control.

### Quantitative RT-PCR Analysis

The mRNA expression levels of 12 selected genes which consistently showed more than twofold difference in two independent microarray analyses and one reference gene, beta-actin, were measured on LightCycler apparatus (Roche, Mannheim, Germany). The same primers as conventional RT-PCR (Table II) were used for quantification. The reaction was performed

**TABLE II. Primer Sets Used for RT-PCR and Quantitative Real Time PCR**

| Gene | Sense primer 5′ → 3′ | Antisense primer 5′ → 3′ |
|---|---|---|
| MAT1A | TGTGCAAGACCGGCATG | TCTCCTCCAGCGTGATG |
| SLC5A6 | TGCCTCTTCAGCGGCTCTC | ACATGGAAAGAACATTCCAA |
| HYOU1 | TGATGGGAAGGGTATCAGGTT | CAACCACTCTACGTGGCTTTCCT |
| TNFRSF12 | TCTTCAGTTCCACAAGTCCGAAGT | GCACTGAAGTCACCGGATGGAA |
| WISP1 | AGACCCACTGAAATGACC | AACCTCCATCTTCCTACC |
| JTB | TAGCAGAAGAGTGCTCTCCAT | GGTCTCTAAGACCCAGGATACAA |
| IGF1R | ATGCTGTTTGAACTGATGCGCA | CCGCTCGTTCTTGCGGCCCCCG |
| PMP22 | TCTGTCCAGGCCACCATGA | GAAGAGTTGGCAGAAGAACAGGA |
| LPL | TGGAGAACTACTCATGTTGAAG | CTGCAAATGAGACACTTTCTC |
| TPM1 | AGCTGGTTGAGGAAGAGTTG | TCGCTCTCAATGATGACCAG |
| LGALS1 | AACCTGGAGAGTGCCTTCGA | GTAGTTGATGGCCTCCAGGT |
| ID2 | AGCCTTCAGTCCGGTGAGGTCC | TCAGACGCCTGCAAGGACAGG |

following the manufacturer's recommendations (Roche). For each run, a standard curve was generated using eight 10-fold serial dilutions of an external standard. The standard was produced by each PCR product, purified using a DNA gel purification kit (Invitrogen, Carlsbad, CA), and quantified by a spectrophotometer. The copy number of each product was calculated using the formula shown below:

Copies/μl = concentration of DNA (ng/μl) $\times 10^{-9} \times 6 \times 10^{23}$/(size of DNA [bp] $\times$ 660). Serially, we diluted standard DNA samples to draw a standard curve, and the copy number of each sample was determined. The ratio of the copy number of a target gene to the beta-actin gene was expressed as a RT-PCR index, and the RT-PCR indexes of the HFD and LFD tumors were compared in the 12 selected genes.

### Immunohistochemistry

Immunohistochemistry of representative tumor sections was performed for insulin-like growth factor I receptor (IGF-IR) using the following protocol. Briefly, deparaffinized and rehydrated sections were steamed for 30 min to enhance antigen retrieval. Immunohistochemical labeling with IGF-IR alfa-antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was performed overnight. Slides were washed with TBS (20 mM Tris-HCl pH 8.0, 0.15 M NaCl) and incubated with a simple stain (Nichirei, Tokyo, Japan) for 60 min at room temperature. After washing with TBST (TBS + 0.1% Tween-20), these were developed with tetrahydrochloride, and counterstained with hematoxylin.

### Human Prostate Cancer

Prostate cancer samples were obtained from 78 patients who underwent radical prostatectomy for PCa at Akita University Hospital from 1998 to 2003. The patient's body mass index (BMI) was obtained from medical records. We assessed IGF-IR immunostaining in both the normal epithelial region and PCa region independently within the same specimen. All evaluation and scoring of immunostaining were performed by N.T. and T.H. who did not know the background of patients. IGF-IR staining intensity in the cytoplasm and membrane was scored on a semiquantitative scale as follows: 0, undetectable; 1, weak; 2, moderate; 3, strong. The fraction of IGF-IR-positive cells was classified based on the percentage of cytoplasmic and membranous IGF-IR in four groups: 0, negative; 1, low expression (0%< to ≤10%); 2, moderate expression (10%< to ≤50%); 3, strong expression (50%<). The total immunoreactivity score was calculated by multiplication of the two values, score = staining intensity × fraction of positive cells [15]. The institutional Review Board of Akita University School of Medicine approved all experiments and human samples were obtained after informed consent.

### Statistical Analysis

All statistical analyses were performed using Microsoft Excel with Statcel 2 software. The significance of serum PSA levels was determined by unpaired Student's *t*-test. Comparison of the rates of tumor growth between the HFD and LFD group was investigated by two-factor repeated measure ANOVA and the relationship between BMI and the immunohistological score was evaluated by one-factor ANOVA. Differences were considered significant at $P < 0.05$.

## RESULTS

### Tumor Growth and Serum PSA Levels in Each Group

Measurable tumors developed in all 20 mice in this experiment and there were no differences in time to the development of a palpable tumor between the groups

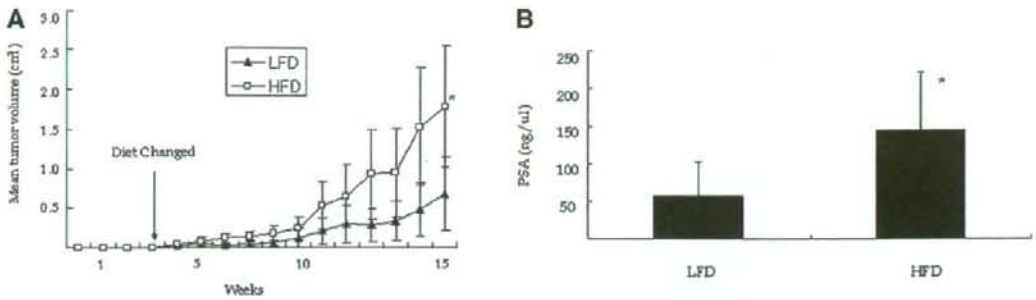


**Fig. 1.** **A**: LNCaP tumor growth in HFD and LFD groups. Statistically significant differences were noted in tumor growth rates between the two groups ($P = 0.001$) by repeated measures ANOVA. **B**: Effect of dietary fat on serum PSA levels. Serum was collected at the time of euthanasia (15 weeks). HFD, serum PSA level of xenograft fed a high fat diet. LFD, serum PSA level of xenograft fed a low fat diet. *$P = 0.006$.

before the start of feeding the HFD or LFD. The HFD and LFD were then started in each group. The final tumor volume in the HFD group ranged from 0.2 to 3.19 ml ($1.79 \pm 0.76$ ml), while that in the LFD group ranged from 0 to 1.25 ml ($0.68 \pm 0.57$ ml). Tumor volumes were significantly greater in the HFD group than in the LFD group ($P < 0.001$, repeated measures ANOVA, Fig. 1A). The mean serum PSA level was significantly higher in the HFD group than the LFD group ($57.9 \pm 45.8$ ng/ml vs. $144.5 \pm 76.2$ ng/ml, $P = 0.006$, Fig. 1B).

### Gene Expression Analyses by Oligonucleotide Microarray

Labeled aRNAs synthesized from each total RNA were hybridized on oligonucleotide arrays. Experiments were repeated twice to minimize experimental errors. After image quantification and normalization of microarray data, we searched for genes with consistent differential expression levels between HFD and LFD groups. After testing the intensities of each gene, we normalized the data (Fig. 2A–C). Genes with less than 100 pixels per spot of expression levels after normalization were omitted from the following analysis. The number of genes with >1.5-fold increase in the expression level in both examinations was 309 (0.89% of total genes on the array), and that of genes with <0.66-fold decrease in the expression level was 225 (0.65%). Among these genes, several genes were related to apoptosis (8 genes), cancer (23 genes), cell cycle (2 genes), cell and development biology (19 genes), extracellular matrix and adhesion molecular (7 genes), biomarker (11 genes), cytokine and inflammatory (15 genes) responses, and signal transduction (29 genes) in the Gene-Ontology database (Table III). Furthermore, 64 genes were found to be consistently up-regulated more than 2.0-fold and 14 genes were down-regulated more than 2.0-fold in the HFD group (Tables IV and V).

### Semi-Quantitative RT-PCR

The validation of differences in gene expression was first screened by semi-quantitative RT-PCR using a distinct batch of RNAs obtained from different xenograft tumor tissues. To examine the reliability of the difference of expression levels detected by profiling analysis using the microarray, RT-PCR analysis with the same RNA samples that had served for microarray analysis was performed. We selected eight up-regulated genes (*MAT1A, SLC5A6, HYOU, TNFRSF12, WISP, JTB, IGF-IR, PMP22*) and four down-regulated genes (*LPL, TPM, LGALS1, ID2*) for further analysis. In agreement with the microarray results, the expression levels of all eight genes, which were considered to be up-regulated by the microarray, were shown to be increased in semi-quantitative RT-PCR (Fig. 3A). The expression levels of five (*MAT1A, SLC5A6, HYOU, TNFRSF12, IGF-IR*) of the eight genes seemed considerably increased. In addition, the expression levels of all four genes, which were considered to be down-regulated by the microarray, were shown to be decreased (Fig. 3B). Among the four genes, the expression level of *LPL* seemed to be significantly reduced.

### Quantitative RT-PCR

For further validation, quantitative RT-PCR analysis was performed to quantity the mRNA levels of the 12 selected genes using an another different batch of RNAs from distinct xenografts. The ratio of the RT-PCR index of the HFD xenograft to that of the LFD xenograft is presented in Figure 3C. Five (*SLC5A6, TNFRSF12, JTB, IGF-IR, PMP22*) of the eight selected genes with

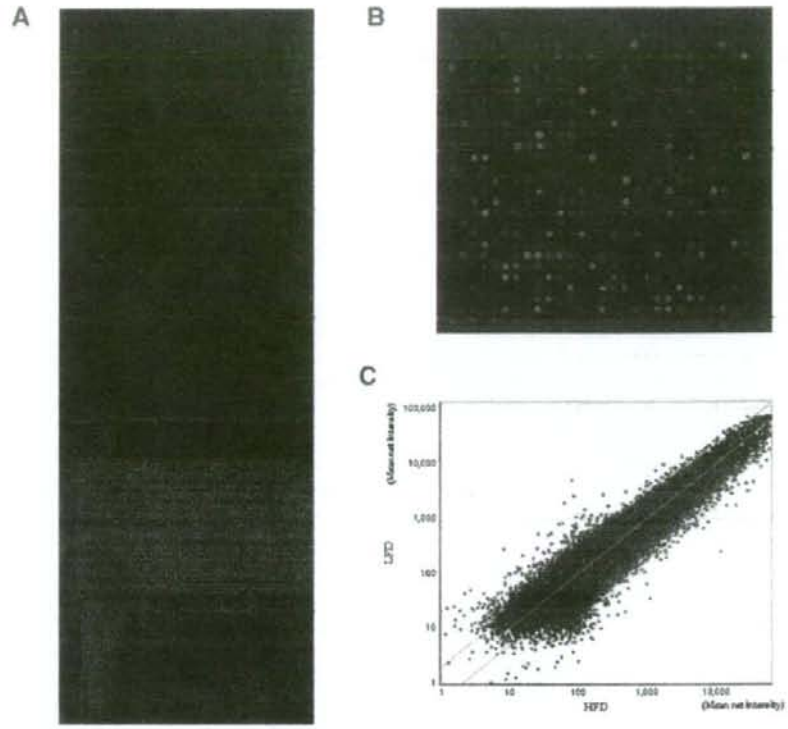


**Fig. 2.** Oligonucleotide microarray analysis. **A**: We used the human 35-K oligonucleotide arrays manufactured by the Operon. Each aRNAs were labeled by Cy5-UTP and fluorescence images of Cy5 dye channels were obtained using a GenePix 4000B scanner. The same experiments were performed twice to increase the reliability. **B**: The signal intensity (red spot) of each spot and its local background, were quantificated and a net intensity by removing the background intensity from the raw intensity was calculated. **C**: The scatter blot shows the expression level of the HFD xenograft in x-axis and the LFD xenograft in Y-axis. Fold differences of the mRNA levels were presented. Red arrow indicated the twofold difference between the two groups.

up-regulated expression in microarray analysis showed more than twofold enhanced expression, while two (*LPL, ID2*) of the four genes had down-regulated expression in microarray analysis of more than twofold decreased expression by quantitative RT-PCR experiment. Among these, the salient difference in the expression level was found in *IGR-IR* (HDF/LFD expression ratio 205.2), *TNFSRF12* (HDF/LFD expression ratio 40.56), and *LPL* (HDF/LFD expression ratio 0.086).

### Immunohistochemistry in Xenograft Tumor

As previous studies have shown the importance of the IGF-I system in the development and progression of PCa [16,17], and significant results in IGF-IR in this study, we further performed immunohistochemical analysis to evaluate the protein expression level. Immunohistochemistry was performed using a tumor xenograft specimen with enhanced growth in the HFD group and a xenograft specimen with slow growth in the LFD group (Fig. 3D). Strong straining in the cytoplasm as well as at the membrane of tumor cells was observed in tumor xenograft tissue in the HFD group, while a weak strain was found in the LFD group (Fig. 3D).

### BMI and IGF-IR Expressions in HumanTissue

As dietary fat intake has been known to affect obesity [18], we finally investigated the relationship between IGF-IR expression in radical prostatectomy specimens and BMI in the corresponding PCa patients. IGF-IR expression was observed variably both in normal epithelial cells and in PCa cells, and the immunoreactivity score (as measured by staining intensity x fraction of positive cells) ranged from 0 to 9 (Fig. 4A–D). Based on the total immunoreactivity score, we divided

**TABLE III. Functional Classification of the Candidate Genes**

| Function[a] | High expression gene[b] | Low expression gene[c] |
|---|---|---|
| Apoptosis | Insulin-like growth factor I receptor | Soluble epoxide hydrolase |
| | Tumor necrosis factor receptor superfamily member Fn14 | |
| | Peripheral myelin protein 22 | |
| | Bcl-2-related protein A1 | |
| | 2-methyl branched chain acyl-CoA dehydrogenase | |
| | 150 kDa oxygen-regulated protein | |
| | Polo-like kinase 1 | |
| Cancer | Insulin-like growth factor I receptor | Soluble epoxide hydrolase |
| | WNT1 inducible signaling pathway protein 1 | Kinesin-like protein KIF2 |
| | Tumor necrosis factor receptor superfamily member Fn14 | MHC class I antigen Cw*7 |
| | Jumping translocation breakpoint protein | Protein kinase A anchoring protein 2 |
| | Peripheral myelin protein 22 | Prokineticin 2 |
| | Seladin-1 | Inhibitor of DNA binding 2, dominant negative Helix-loop-helix protein |
| | Bcl-2-related protein A1 | Protein kinase C, beta type |
| | Scatter factor | Transforming growth factor alpha |
| | Aminopeptidase N | Tropomyosin 1 alpha chain |
| | Cell division protein kinase 5 | |
| | 150 kDa oxygen-regulated protein | |
| | Nuclear receptor coactivator 4 | |
| | Cytochrome c-1 | |
| | Polo-like kinase 1 | |
| Cell cycle | Bcl-2-related protein A1 | Soluble epoxide hydrolase |
| Cell and development biology | Insulin-like growth factor I receptor | Glycogen synthase kinase-3 beta |
| | WNT1 inducible signaling pathway protein 1 | Alpha 2 actin |
| | Tumor necrosis factor receptor superfamily member Fn14 | MHC class I antigen Cw*7 |
| | Muscle-cadherin | Prokineticin 2 |
| | Bcl-2-related protein A1 | B-cell specific transcription factor |
| | Hepatopoeitin A | Interleukin 1 family member 9 |
| | T cell-specific protein P228 | Platelet membrane glycoprotein Iib |
| | Aminopeptidase N | Transcription factor SOX-10 |
| | Hemoglobin beta chain | inhibitor of DNA binding 2, dominant negative Helix-loop-helix protein |
| | | Transforming growth factor alpha |
| Extracellular matrix and adhension molecules | Tumor necrosis factor receptor superfamily member Fn14 | Prokineticin 2 |
| | Hepatopoeitin A | Platelet membrane glycoprotein Iib |
| | Aminopeptidase N | Transforming growth factor alpha |
| | | Kinesin-like protein KIF2 |
| Biomaker | Insulin-like growth factor I receptor | A-kinase anchor protein 2 |
| | WNT1 inducible signaling pathway protein 1 | Platelet membrane glycoprotein Iib |
| | Cell division protein kinase 5 | Protein kinase C, beta type |
| | Nuclear receptor coactivator 4 | Transforming growth factor alpha |
| | Cytochrome c-1 | Tropomyosin 1 alpha chain |
| | | Glycogen synthase kinase-3 beta |
| Cytokine and inflamatory response | Bcl-2-related protein A1 | Janus kinase 2 |
| | Small inducible cytokine A5 | MHC class I antigen Cw*7 |
| | Aminopeptidase N | 36 kDa phospho-tyrosine adaptor protein |
| | Hemoglobin beta chain | B-cell specific transcription factor |
| | Alpha-1-acid glycoprotein 2 | Interleukin 1 family member 9 |
| | | Platelet membrane glycoprotein Iib |
| | | Serine/threonine protein phosphatase 5 |
| | | DNA-binding protein inhibitor ID-2 |
| | | Protein kinase C, beta type |
| | | Transforming growth factor alpha |

*(Continued)*

**TABLE III. (*Continued*)**

| Function[a] | High expression gene[b] | Low expression gene[c] |
|---|---|---|
| Signal transduction | Insulin-like growth factor I receptor | Peptidyl-prolyl *cis–trans* isomerase |
| | 5-aminolevulinic acid synthase | Glycogen synthase kinase-3 beta |
| | WNT1 inducible signaling pathway protein 1 | Adenylate cyclase-inhibiting G alpha protein |
| | Peripheral myelin protein 22 | Janus kinase 2 |
| | Bcl-2-related protein A1 | MHC class I antigen Cw*7 |
| | Hepatopoeitin A | Metallothionein-IG |
| | Metabotropic glutamate receptor 2 | Metabotropic glutamate receptor 1 |
| | Small inducible cytokine A5 | Paired box protein Pax-5 |
| | Monocyte to macrophage differentiation protein | Interleukin 1 family member 9 |
| | Cell division protein kinase 5 | Platelet membrane glycoprotein IIb |
| | Hemoglobin beta chain | Serine/threonine protein phosphatase 5 |
| | Alpha-1-acid glycoprotein 2 | Taste receptor type 2 member 7 |
| | Polo-like kinase 1 | Inhibitor of DNA binding 2, dominant negative Helix-loop-helix protein |
| | Death-associated protein kinase 3 | Protein kinase C, beta type |
| | | Transforming growth factor alpha |

[a]Functional classification was performed using a Microarray Data Analysis Tool ver 1.0 (supplied by Filgen, Inc.), which was based on a Gene-Ontology data base.
[b]Genes with >1.5-fold change in expression in the microarray analysis.
[c]Genes with <0.66-fold change in expression in the microarray analysis.

78 patients into three groups, which were low (scored as 0, 1, or 2), moderate (scored as 3 or 4), and high (scored as 6 or 9). The averages of BMI in three groups in normal epithelial cells were $22.94 \pm 2.52$, $24.35 \pm 2.90$, and $24.29 \pm 2.75$, while those in PCa cells were $23.43 \pm 2.33$, $24.31 \pm 3.40$, and $23.96 \pm 2.58$, respectively (Fig. 4E). Although there was no significant difference in the mean BMI level among these three groups in both normal epithelial and PCa cells ($P = 0.1252$ and $P = 0.5030$, respectively), patients with low IGF-IR expression in normal or PCa cells tended to have a lower BMI level than those of other groups with higher IGF-IR expression (Fig. 4E).

## DISCUSSION

In support of previous studies [10], our results confirmed a significant difference in tumor volume, growth rate and serum PSA level after treatment with HFD and LFD in the LNCaP xenograft model.

Wang et al. [10] found that the tumor growth rate, final tumor weight, ratio of final tumor weight to animal weight and serum PSA levels were substantially highest in the group that continued with the highest percentage of corn oil fat diet than those of other groups. Furthermore, Ngo et al. also showed that LAPC-4 xenograft treated with HFD had significantly higher tumor growth in a model with equal caloric intake of each [12]. In a study which employed three different unsaturated fatty acids, Connolly et al. [9] demonstrated that tumor weight in the DU145 xenograft in mice with low oleic and linoleic acid feeding was significantly lower than that in mice in the conventional corn and linseed oil-rich group.

Unsaturated fatty acids are classified into three types: a mono-unsaturated, omega-3 polyunsaturated and omega-6 polyunsaturated fatty acid, and different metabolic pathways are involved. The safflower oil used in the present study is composed of oleic acid, which is a mono-unsaturated acid, while the corn oil used by Ngo et al. is composed of linoleic acid, which is an omega-6 polysaturated fatty acid. Ngo et al. [19] claimed that the arachidonic acid driven from linoleic acid is eventually converted to prostaglandin E2, which is believed to play an important role in the growth of PCa. Therefore one of their hypotheses is that fat consumption might affect tumor growth via the arachidonic cascade. On the other hand, in support of the study by Collony et al., our study indicated that PCa cells may be promoted not only by linoleic acid and omega-6 fatty acid, but also by oleic acid; therefore, we suggest that the mechanism except for the arachidonic cascade driven from linoleic acid may affect the growth of PCa cell lines under high fat feeding.

For the precise molecular mechanisms underlying the enhanced growth of PCa cells under high fat feeding, we employed microarray analysis to screen genes with different expression levels in tumor xenografts under HFD and LFD. Microarray analysis revealed 309 up-regulated genes with >1.5-fold difference