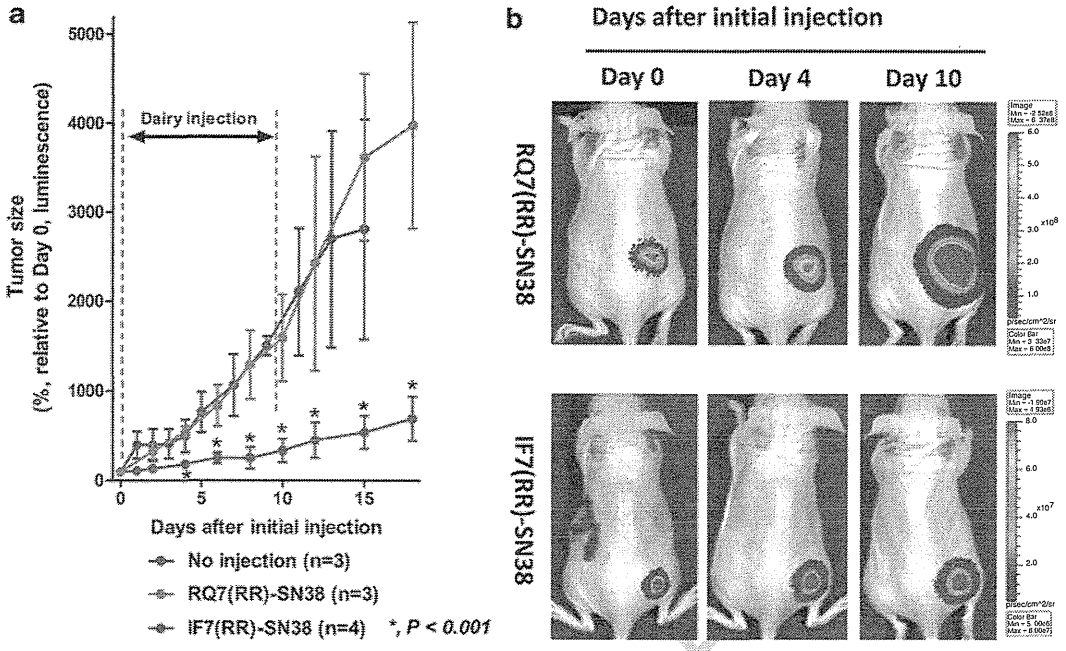

[AU1] **Fig. 8** Anticancer effect of IF7(RR)-SN38 on a small tumor. IF7(RR)-SN38 or RQ7(RR)-SN38 were injected into HCT116-Luc tumor bearing mice (starting from day 10 after tumor implantation) and photon numbers were measured every 2–3 days. Tumor-bearing mice were administered 0.81 μmol/kg (1.74 mg/kg) of IF7(RR)-SN38 from days 23 to 43 (20 times). In the IF7(RR)-SN38 group, tumor growth was significantly suppressed, whereas tumor growth in control mice injected with RQ7(RR)-SN38 remained unchanged (Fig. 7a). Representative photon images are shown in Fig. 7b

4 Notes

1. *Animal care and protocol approval.* Animal care and pain management using anesthesia and analgesia are crucial components of protocols using animals. All experiments must conform to the Principles of Laboratory Animal Care and the Guide for Care and Use of Laboratory Animals. Animal experiment protocols should be approved by the institutional animal care and use committee.
2. The data suggest the interaction between Anxa1 and heparan sulfate on cell surface (Fig. 2b). Because it is evident that heparan sulfate regulates tumorigenesis, progression and metastasis [7, 8], the elongation of heparan sulfate by glycosyltransferases (EXT1/EXT2) has potential to play a key role for cancer biology (Fig. 2c).
3. *Anesthesia in mice.* Tribromoethanol is the standard anesthetic agent used in mice. It produces short-term (15–30 min) surgical anesthesia with good muscle relaxation and moderate respiratory depression. It was once manufactured specifically for use

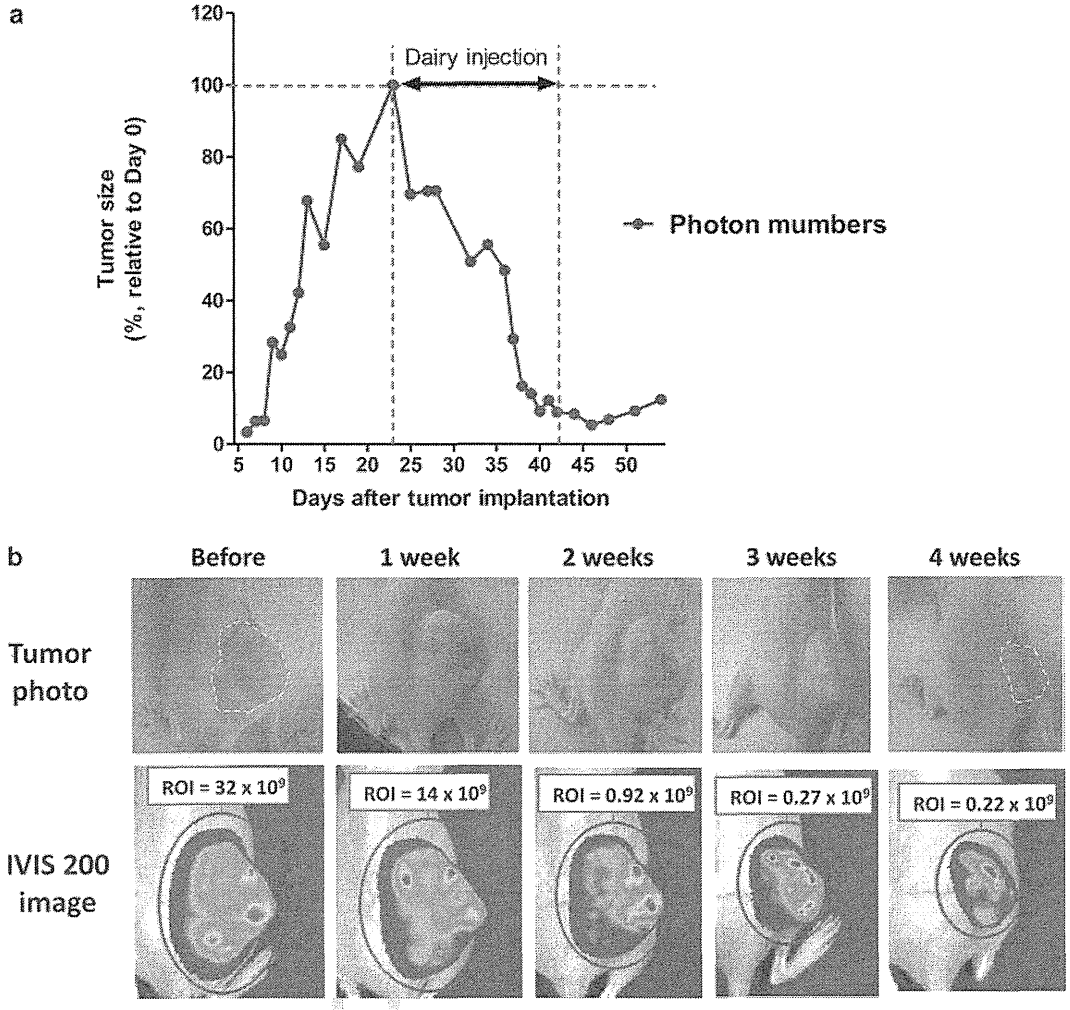

Fig. 9 Anticancer effect of IF7(RR)-SN38 on a large tumor. IF7(RR)-SN38 was injected into HCT116-Luc tumor bearing mice (starting from day 23 after tumor implantation) and photon numbers were measured every 2–3 days. Tumor-bearing mice were administered 6.5 μmol/kg (13.9 mg/kg) of IF7(RR)-SN38 from days 23 to 43 (20 times). Photon numbers and tumor size were dramatically reduced after IF7(RR)-SN38 injection (Fig. 8a, b)

Table 1 t1.1
Dosage of SN-38 used in the experiments t1.2

	Anticancer drug	Dosage per single injection	Number of injection	Total dosage	
Meyer-Losic et al. [8]	Irinotecan	30 μmol/kg (95 mg/kg)	3 times	90 μmol/kg	t1.4 t1.5
Our study	IF7(RR)-SN38	6.5 μmol/kg (13.9 mg/kg) 0.81 μmol/kg (1.74 mg/kg)	20 times for large time 10 times for small tumor	130 μmol/kg 8.1 μmol/kg	t1.6 t1.7

It is notable that the IF7-SN38 injections administered in this study contained 0.81 μmol/injection (1.74 mg/kg) or 6.5 μmol/kg (13.9 mg/kg), whereas the effective dose of SN-38 conjugated with a non-tumor vasculature targeting peptide was reported to be 95 mg/kg in a previous study [11] t1.8 t1.9 t1.10

as an anesthetic under the name Avertin®. However, this product is no longer commercially available. Investigators who wish to use tribromoethanol as an anesthetic must make their own solution. A stock solution of tribromoethanol is made by mixing equal amounts of tribromyl ethyl alcohol and tertiary amyl alcohol. This must be stored at 4 °C in the dark for no longer than 1 year. A working solution must be prepared each time by diluting the stock solution to 1.25 % using distilled water or saline. Because this agent has toxic degradation products, the technician should only use a freshly mixed solution or one that has been stored for no more than 1–2 weeks at 4 °C in the dark. Intraperitoneal injection of 400–500 μL of a 1.25 % working solution will provide adequate anesthesia for surgical experiments in mice.

4. *Disinfection of mice.* In order to prevent bacterial infection, all invasive procedures should be performed using aseptic techniques, especially when immunodeficient mice are used. Before attempting to introduce any instrument or agent into an animal's body, the injection or inoculation site should be cleansed and disinfected with 70 % ethanol or an antiseptic agent.
5. It is important to remove all thin membranes (epidermis, dermis, subcutis, and cutaneous muscle) to avoid edematous areas and to thin the skin.
6. The coverslip should be placed on the front of the frame before it is fixed to the mouse.
7. The top and bottom of the chamber should be ligated to hold the mouse in position, but care should be taken to avoid making the ligature too tight.
8. It is not easy to create suitable conditions for a skinfold chamber window. It is better to prepare 1.5–2-fold the number of mice required for the experiments.
9. It is important to take a small piece of the tumor that enables visualization of tumor vasculature.
10. Use fine forceps to hold the needle. For tail vein injection, mice should be older than 6 weeks of age because at a younger age their blood vessels are not thick enough. The most important part of the procedure is the method of holding the mouse because injection needs accurate manipulation of the needle. Three blood vessels are visible on the back of a mouse's tail: a central artery and a vein on each side. The best syringe for tail vein injection is one used for insulin injection with a 28G needle. The procedure for tail vein injection requires careful handling of the mouse and needle. Repeated practice is essential for success with this technique. For more information, please read the reference [15].

11. The catheter can easily be occluded by blood clots. To avoid clotting, flush the catheter occasionally.
12. For repeat experiments, adjust the zero point every time.
13. After 10 min, irradiation of specimens with a UV lamp is limited to only those times when photos are taken in order to avoid fluorescence bleaching.
14. The IF7(RR)-SN38 group displayed significantly suppressed tumor growth compared with the control and RQ7(RR)-SN38 group mice (Fig. 8a, b).

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Original Article

Distinct Cancer-Specific Survival in Metastatic Prostate Cancer Patients Classified by a Panel of Single Nucleotide Polymorphisms of Cancer-Associated Genes

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Abstract

Individual genetic variations may have a significant influence on the survival of metastatic prostate cancer (PCa) patients. We aimed to identify target genes and their variations involved in the survival of PCa patients using a single nucleotide polymorphism (SNP) panel. A total of 185 PCa patients with bone metastasis at the initial diagnosis were analyzed. Germline DNA in each patient was genotyped using a cancer SNP panel that contained 1,421 SNPs in 408 cancer-related genes. SNPs associated with survival were screened by a log-rank test. Fourteen SNPs in 6 genes, *XRCC4, PMS1, GATA3, IL13, CASP8*, and *IGF1*, were identified to have a statistically significant association with cancer-specific survival. The cancer-specific survival times of patients grouped according to the number of risk genotypes of 6 SNPs selected from the 14 SNPs differed significantly (0-1 v. 2-3 v. 4-6 risk genotypes; $P = 7.20 \times 10^{-8}$). The high-risk group was independently associated with survival in a multivariate analysis that included conventional clinicopathological variables ($P = 0.0060$). We identified 14 candidate SNPs in 6 cancer-related genes, which were associated with poor survival in patients with metastatic PCa. A panel of SNPs may help predict the survival of those patients.

Keywords

prostate cancer, bone metastasis, survival, single nucleotide polymorphism

Introduction

Over the past few decades, a great deal of effort has been devoted to identifying the genes involved in prostate cancer (PCa), and recent genome-wide association studies using the array technique have identified several dozen promising candidate genes associated with PCa risk.[1-5] Most of the past studies attempted to identify genes involved in PCa susceptibility, while more recent studies have begun to consider the clinical significance of single nucleotide polymorphisms (SNPs) as predictive or prognostic markers.[6] Accumulating data suggested that interindividual genetic variations represented by SNPs might partially affect the prognosis of PCa as well as the biological characteristics of the cancer itself by modulating the sensitivity to endocrine therapy or chemotherapy and the microenvironment around cancer cells.[6] It is of clinical importance to identify such SNPs for the future optimization of individualized treatment plans.

We previously demonstrated that the SNPs of *insulin-like growth factor 1* (*IGF1*) and *cytochrome P450 19* (*CYP19*), which had earlier been implicated in PCa susceptibility,[7,8] might be markers for poor prognosis in patients with metastatic PCa.[9] However, in most of the past studies, only a small number of genes were evaluated for their association with survival as a supportive analysis for a larger case-control study, and there have been few

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comprehensive SNP investigations based on this concept. Recently, several studies searched for SNPs related to the survival of lung and bladder cancer patients and reported some candidate SNPs or genes.[10-12] Meanwhile, in PCa, although a few studies tried to explore survival-associated SNPs in a large population-based cohort,[13,14] there was no study that exploratively investigated SNPs involved in the survival of patients with a specific disease status.

In this study, we conducted an exploratory study using a cancer SNP panel to identify SNPs associated with the survival of PCa patients with bone metastasis at initial presentation.

Results

Clinicopathological background of patients. The patient demographics and clinical characteristics are shown in Table 1. Eighteen patients received at least one course of docetaxel or docetaxel-containing chemotherapy during the follow-up period or before death. The median overall survival and cancer-specific survival times in the whole patient group were 4.62 and 4.77 years, respectively.

Survival analysis. We narrowed the number of SNPs down to 176 at the first screening step using the minimum genotype frequency (MGF), and the top 14 SNPs were finally selected at the second screening step based on the false discovery rate (FDR) criterion (Table 2). Hardy-Weinberg equilibrium was assumed for all the SNPs. The selected SNPs were located in 6 genes: *X-ray repair complementing defective repair in Chinese hamster cells 4* (*XRCC4*), *postmeiotic segregation increased 1* (*PMS1*), *GATA binding protein 3* (*GATA3*), *interleukin 13* (*IL13*), *caspase 8* (*CASP8*), and *IGF1* (Table 2). The SNPs showed strong linkage disequilibrium with each other in 4 of 6 genes with multiple candidate SNPs (D′ = 1.000 in *XRCC4* and *IL13*, 0.985-1.000 in *PMS1*, and 0.9862-1.000 in *GATA3*).

In the leave-one-out cross-validation (LOOCV) analysis for predicting cancer-specific survival, a total of 165 patients were classified into high- and low-risk groups due to missing values of at least one SNP locus in 20 patients. The median cancer-specific survival time of the high-risk group was significantly shorter than that in low-risk patients (4.29 v. 7.09 years, respectively; $P = 0.0050$) (Fig. 1).

In a univariate Cox proportional hazard analysis of the association between cancer-specific survival and the clinicopathological variables, a higher alkaline phosphatase (ALP) level (hazard ratio [HR] = 2.92; 95% confidence interval [CI] = 1.79-4.77; $P = 2.05 \times 10^{-5}$), higher lactate dehydrogenase (LDH) level (HR = 2.70; 95% CI = 1.47-4.95; $P = 1.34 \times 10^{-3}$), and Gleason score of 9 or greater (HR = 2.44; 95% CI = 1.52-3.90; $P = 2.01 \times 10^{-4}$) were significantly associated with a shorter cancer-specific survival time (Table 3). The administration of docetaxel or docetaxel-containing chemotherapy did not significantly influence cancer-specific survival ($P = 0.369$). In the LOOCV analysis, the effect of the predicted risk classification based on genetic variables was statistically significant after adjusting for the clinicopathological variables ($P = 0.0060$ for the Wald statistic in the multivariate Cox regression model evaluated by the permutation method). These results suggest that the genetic classification was independent of the clinical prognostic variables.

Next, we performed a survival analysis according to the number of risk genotypes within the 6 candidate genes. Since all the SNPs in a gene were in strong linkage disequilibrium to each other, the SNP with the smallest P value based on a log-rank test was picked to represent each gene (i.e., rs2891980 for *XRCC4*, rs256550 for *PMS1*, rs570730 for *GATA3*, rs1295686 for *IL13*, rs2293554 for *CASP8*, and rs2162679 for *IGF1*). We gave the same weight to each SNP because the HRs of risk genotypes in the representative SNPs were within the range of 1.71 to 2.00. The developed score based on the refined set of 6 SNPs was highly correlated with the original score based on the 14 SNPs (Spearman correlation: 0.88).

Table 1. Patient Demographics and Clinical Characteristics.

Variable	Mean ± standard deviation	Median	Range
Age, y	69.4 ± 8.8	70	45-89
Follow-up, mo	43.8 ± 33.4	37	1-210
PSA, ng/mL	1,088 ± 1,980	313	0.2-12,490
Hemoglobin, g/dL	13.3 ± 2.0	13.5	6.2-17.4
ALP, IU/L	654 ± 902	306	7-5,870
LDH, IU/L	299 ± 184	232	97-956

Variable	*n*	%
Gleason score		
<6	15	8.1
7	26	14.1
8	39	21.1
9	81	43.8
10	13	7.0
Unknown	11	5.9
Site of metastases		
Bone alone	95	51.3
Bone + lymph nodes	89	48.1
Bone + visceral organs	13	7.0
Initial treatments		
Surgical castration alone	22	11.9
LH-RH analog alone	47	25.4
Combined androgen blockade	116	62.7

Note: ALP = alkaline phosphatase; LDH = lactate dehydrogenase; LH-RH = luteinizing hormone–releasing hormone; PSA = prostate-specific antigen.

Table 2. SNPs Selected for Association with Cancer-Specific Survival of Prostate Cancer with Bone Metastasis.

Gene (location, function), SNP	Risk genotypes	MGF[a]	Log-rank χ^2	*P*
XRCC4 (5p14, DNA strand-break repair)				
rs2891980	GG (v.AG,AA)	45	9.49	0.0021
rs1805377	AA (v.AG, GG)	46	9.10	0.0026
PMS1 (2q31, DNA mismatch repair)				
rs256550	GG,AG (v.AA)	46	8.39	0.0038
rs256552	GG,AG (v.AA)	46	7.82	0.0052
rs256564	AA,AG (v. GG)	46	7.27	0.0070
rs256563	GG,AG (v.AA)	46	6.84	0.0089
rs256567	AA,AG (v. GG)	44	6.04	0.0140
GATA3 (10p14, transcription factor)				
rs570730	GG,AG (v.AA)	50	6.86	0.0088
rs10752126	GG, CG (v. CC)	49	6.85	0.0089
rs569421	GG,AG (v.AA)	49	5.21	0.0225
IL13 (5q31, cytokine)				
rs1295686	AA,AG (v. GG)	50	6.22	0.0126
rs20541	AA,AG (v. GG)	48	6.06	0.0138
CASP8 (2q33, apoptosis)				
rs2293554	AA (v.AC, CC)	48	5.46	0.0195
IGF1 (12q23, growth factor)				
rs2162679	GG,AG (v.AA)	45	5.45	0.0196

Note: MGF = minimum genotype frequency; SNP = single nucleotide polymorphism.
[a]MGF was defined as the lower frequency of 2 dichotomized genotype groups in each SNP.

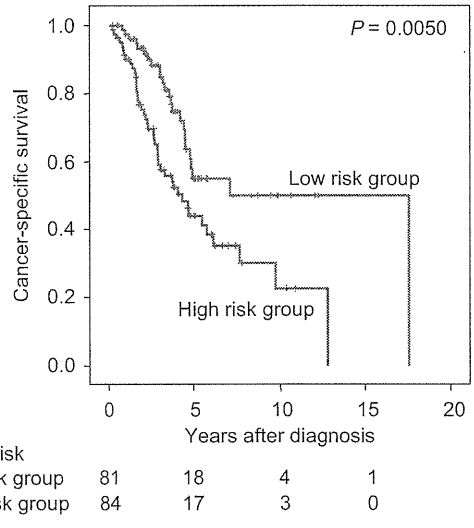

Figure 1. Cancer-specific survival of patients according to risk categorization using candidate SNPs identified by array analysis. A prognostic scoring index using the 14 SNPs selecz the screening was developed by incorporating the difference in their effect sizes to classify high-risk and low-risk groups. Its predictive accuracy was assessed by the LOOCV analysis for the whole model building process. The median cancer-specific survival time of the high-risk group was significantly shorter than that in low-risk patients ($P = 0.0050$).

Finally, using the entire set of patients, we developed a multivariate model for prognostic prediction using both the genetic score and the clinical prognostic factors to establish a method of independent, external validation for future studies. Each patient was assigned to 1 of 3 groups: a favorable-risk group with 0 to 1 risk genotypes, an intermediate-risk group with 2 to 3 risk genotypes, and a poor-risk group with 4 to 6 risk genotypes based on the genetic score using 6 SNPs. The cancer-specific survival times were significantly different among the 3 groups, with median values of 13.3, 7.0, and 3.8 years for the favorable-, intermediate-, and poor-risk groups, respectively (log-rank, $P = 7.20 \times 10^{-8}$) (Fig. 2). In the multivariate Cox proportional hazard analysis with this model, the risk classification (poor v. favorable or intermediate risk) (HR = 3.06; 95% CI = 1.80-5.19; $P = 3.58 \times 10^{-5}$) was again indicated as an independent variable, predicting cancer-specific survival along with the ALP level (HR = 2.22; 95% CI = 1.32-3.73; $P = 2.63 \times 10^{-3}$) and Gleason score (HR = 2.16; 95% CI = 1.31-3.56; $P = 2.69 \times 10^{-3}$) (Table 3). For subgroup analyses (Fig. 3), patients were divided into subgroups on the basis of ALP levels (<350 IU/L or ≥350 IU/L) and Gleason scores (<9 or ≥9). Then, cancer-specific survival times were compared in each subgroup stratified by the number of risk genotypes (0-3 v. 4-6). In the subgroup with ALP <350 IU/L, the cancer-specific survival time for patients with 4 to 6 risk genotypes was significantly worse than that for patients with 0 to 3 risk genotypes ($P = 1.69 \times 10^{-7}$). No significant difference in survival was observed between patients with 0 to 3 risk genotypes and those with 4 to 6 risk genotypes in the subgroup with ALP ≥350 IU/L ($P = 0.681$). With regard to Gleason scores, there were significant differences in cancer-specific survival between patients with 0 to 3 risk genotypes and those with 4 to 6 risk genotypes in

Table 3. Cox Proportional Hazard Regression Analysis of Factors Associated with Cancer-Specific Survival.

	Univariate analysis		Multivariate analysis (model 1)		Multivariate analysis (model 2)	
Variable	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (≥70 v. <70 y)	1.34 (0.85-2.11)	0.204				
PSA (≥315 v. <315 ng/mL)	1.42 (0.90-2.24)	0.137				
Hemoglobin (≤13.5 v. >13.5 g/dL)	1.14 (0.71-1.83)	0.589				
ALP (≥350 v. <350 IU/L)	2.92 (1.79-4.77)	2.05×10^{-5}	2.64 (1.51-4.62)	6.93×10^{-4}	2.22 (1.32-3.73)	2.63×10^{-3}
LDH (≥500 v. <500 IU/L)	2.70 (1.47-4.95)	1.34×10^{-3}	1.55 (0.78-3.10)	0.215	1.22 (0.62-2.40)	0.570
Gleason score (≥9 v. <9)	2.44 (1.52-3.90)	2.01×10^{-4}	2.10 (1.20-3.67)	8.95×10^{-3}	2.16 (1.31-3.56)	2.69×10^{-3}
Risk group[a] (high v. low)	2.13 (1.28-3.52)	3.43×10^{-3}	2.07 (1.18-3.62)	1.13×10^{-2}		
No. of risk genotypes[b] (4-6 v. 0-3)	3.21 (2.04-5.05)	4.70×10^{-7}			3.06 (1.80-5.19)	3.58×10^{-5}

Note: ALP = alkaline phosphatase; CI = confidence interval; HR = hazard ratio; LDH = lactate dehydrogenase; PSA = prostate-specific antigen.
[a]Group was classified by a leave-one-out cross-validation method.
[b]The number of risk genotypes was calculated using 6 representative single nucleotide polymorphisms selected from each candidate gene.

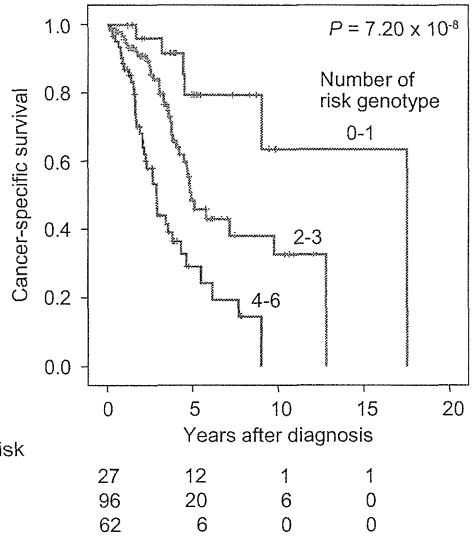

Figure 2. Cancer-specific survival of patients according to the number of risk genotypes of 6 representative SNPs selected from 6 candidate genes. Each patient was assigned to 1 of 3 groups according to the number of risk genotypes in 6 representative SNPs selected from each candidate gene: 0-1, 2-3, and 4-6. The cancer-specific survival times differed significantly among the 3 risk groups ($P = 7.20 \times 10^{-8}$).

both Gleason score subgroups (<9: $P = 0.0004$; ≥9: $P = 0.0004$).

The same exploratory approach was performed for selecting SNPs associated with overall survival. However, none of the SNPs met the screening criteria of MGF and FDR employed in this study.

Discussion

A comprehensive SNP analysis was performed to identify SNPs related to the survival of metastatic PCa patients using a cancer-related SNP panel on which SNPs in various types of genes related to cell cycle regulation, apoptosis, DNA repair, proliferation, migration, steroid metabolism, among others, were integrated. Based on the results of this analysis, we selected 14 candidate SNPs in 6 genes and showed that they had the potential to increase the accuracy of survival prediction. Among the 6 genes involved, 2 were related to DNA repair (*XRCC4* and *PMS1*), and the others were involved in the regulation of a transcription factor (*GATA3*), immune system (*IL13*), apoptosis (*CASP8*), and cell growth (*IGF1*). Most of the SNPs were mapped on either the intron or 3′-untranslated region, and one of the SNPs (rs1805377 in *XRCC4*) altered a splicing acceptor site. Meanwhile, only one exonic SNP (rs20541 in *IL13*) comprised a nonsynonymous amino acid substitution, which changed a glutamine to an arginine at position 144 (144 Glu>Arg).

The aim of this study was to identify candidate SNPs and cancer-related genes involved in the survival of PCa patients with bone metastasis. Although the percentage of patients who are diagnosed with PCa with distant metastasis has decreased to as low as 1% in countries where prostate-specific antigen (PSA) screening is widely prevalent,[15] we expect the present results to have clinical significance not only for such patients but also potentially for patients with locally advanced or recurrent PCa. One of the goals of this study was to actively intervene in the therapy to prolong survival by the individual genomic background based on progression-related SNPs in addition to conventional markers. However, it is to be noted that the SNPs in the 6 genes selected in this study are not candidates for predictive markers but prognostic markers at present. To optimize the treatment using this panel of SNPs, there will be a need for further investigations in which the responses to particular treatments are compared between patients with different genotypes along with sufficient validation studies using a large-scale sample set. The other goal was to identify genes

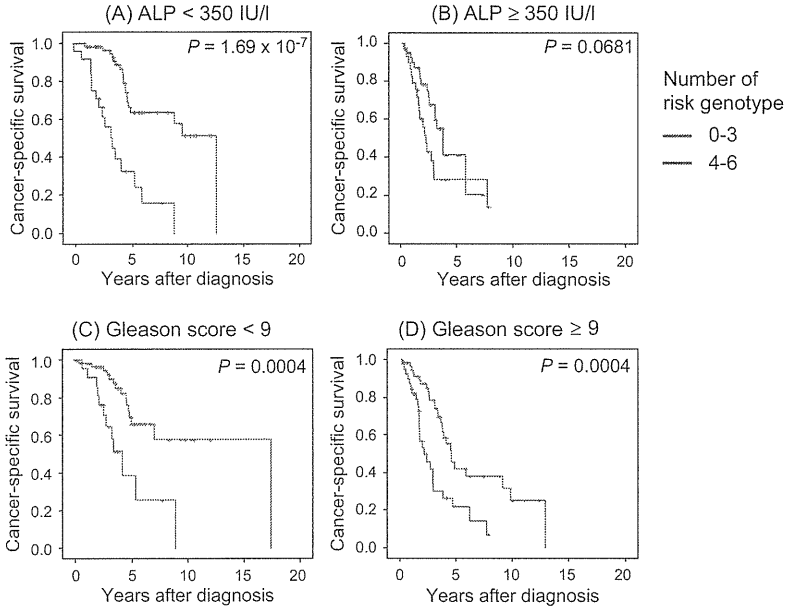

Figure 3. Cancer-specific survival based on the number of risk genotypes for 6 representative SNPs in the following groups: (**A**) ALP <350 IU/L, (**B**) ALP ≥350 IU/L, (**C**) Gleason score <9, and (**D**) Gleason score ≥9. There were significant differences in cancer-specific survival between patients stratified by the number of risk genotypes (0-3 v. 4-6) in the subgroups shown: (**A**) $P = 1.8 \times 10^{-7}$, (**C**) $P = 0.0004$, and (**D**) $P = 0.0004$.

involved in PCa progression. We believe that revealing the function of these genes will help to delineate the molecular mechanism underlying the progression of PCa as well as to the development of therapeutic agents targeting those genes.

We consider that a cohort of metastatic PCa patients is one of the most suitable clinical models for assessing the individual genetic background of cancer progression. To assess the influence of inherited genetic factors on cancer progression, several cohorts of patients with different clinical backgrounds can be chosen for the analysis. In patients with localized PCa, there are many treatment options, and it can be difficult to distinguish the results due to genetic factors and those due to treatment-specific factors. Meanwhile, although there was a more than 10-year gap in the entry period in this study, the patients with metastatic PCa at diagnosis were treated according to a fairly uniform therapeutic protocol consisting of androgen suppression therapy followed by estrogens, steroids, and chemotherapeutic agents. Although patients with castration-resistant PCa are often treated with docetaxel, it is suggested that this agent had only a limited effect on survival in this study design.[16]

Using the cohort as a clinical model for assessing cancer progression, various biological mechanisms are speculated to explain why each candidate SNP affects survival.[6] First, the SNP may affect the cancer biology, including cell cycle regulation, tumor transformation, apoptosis, cell adhesion, or migration. The SNPs directly relevant to cancer biology may influence patient survival as well as a susceptibility or clinical phenotype of cancers. Second, the SNPs may affect the response to endocrine therapies or chemotherapies and adverse effects. Recent genetic and pharmacological studies have revealed that many SNPs in genes involved in drug and hormone metabolism and disposition exerted an influence on circulating or intracellular levels of drugs or hormones.[17,18] Furthermore, the response to cytotoxic agents is also considered sensitive to genes other than drug-related genes, such as DNA repair genes. The clinical response modulated by functional SNPs of those genes may affect survival. Thirdly, the SNPs may have an effect on the intracorporeal environment and microenvironment around cancer cells, thus affecting the invasive and metastatic potential.[19] Furthermore, intracrine androgen levels that may be affected by variants of steroid hormone-related genes in the prostate might play a role in the growth of castration-resistant cancer cells.[20]

We acknowledge that this study has several limitations. First, the possibility of patient selection bias cannot be eliminated due to its retrospective multi-institutional study design. The majority of the patients enrolled in this study are incidence cases, but the possible inclusion of prevalence cases could have affected the results. Such a bias could have led to a slightly better survival time than previously reported by eliminating patients with extremely short survival times. Second, if such patients were excluded from the analysis, less frequent but more important SNPs related to the extremely aggressive phenotype could have slipped out of the screening for candidate SNPs. To explore such rare SNPs, a higher statistical power with a larger number of carefully selected samples is needed. In this study with a relatively small number of patients, a higher cutoff of MGF was required to achieve sufficient statistical power. Thus, quite common SNPs that have a modest influence on survival were considered to be detected in this study. Thirdly, the validation of the results was still insufficient due to the lack of the number of patients. Additionally, patients enrolled in this study were entirely from Japan. The expected prevalence of SNPs will differ relative to other

populations. External validation with a larger series of patients or other ethnicities will be required to translate these results into clinical applications. A multi-institutional prospective trial for validating the candidate SNPs as prognostic markers for PCa patients with bone metastasis (UMIN trial ID: UMIN000009785) is now ongoing by us and other members in Japan.

In conclusion, we identified 14 candidate SNPs in 6 cancer-related genes associated with cancer-specific survival (CSS) in PCa patients with bone metastasis at the initial diagnosis. Using a panel of the SNPs, the prediction of the survival and optimization of the individualized treatment for patients with advanced PCa may be possible in the future.

Materials and Methods

Patients. From July 1980 to September 2008, 191 native Japanese patients with PCa with bone metastasis at initial presentation were enrolled in this study. They were diagnosed at Akita University Hospital and its related community hospitals, Kyoto University Hospital, Tohoku University Hospital, and Hirosaki University Hospital, and had undergone no previous treatments. Pathological diagnosis was made by prostate needle biopsy, and metastasis was identified by radiography, computed tomography, or bone scintigraphy. All the patients were initially treated by endocrine therapy. Combined androgen blockade was defined as luteinizing hormone–releasing hormone analog plus antiandrogens. After treatment failure with the primary therapy, optional therapies, including other antiandrogens, estrogens, steroids, chemotherapeutic agents, or palliative radiation, were added to or substituted for the preceding therapies.

Pathological grading of needle biopsy specimens was performed by local pathologists. Pretreatment hemoglobin, ALP, LDH, and PSA levels before the initial treatment of PCa were obtained from medical charts. An independent end-point reviewer at each institution determined the cause of death on the basis of standardized extractions from the patient files without providing genotype data of each patient.

Genotyping analysis. Approximately 2 ug of genomic DNA was prepared from a peripheral blood sample of each patient using a QIAamp Blood Kit (Qiagen, Hilden, Germany) or standard phenol-chloroform extraction. Genotyping was performed using a Cancer SNP Panel (Illumina Inc., San Diego, CA) that contained 1,421 SNPs in 408 cancer-related genes selected from the SNP500 Cancer Database.[21] This panel included several genes related to the synthesis or metabolism of androgens and steroid hormones. Since 6 of 191 samples were eliminated from this study due to unsuccessful genotyping (sample call rate: 96.9%), a total of 185 patients were subjected to statistical analyses. The locus success rate and overall genotype call rate were 98.2% and 95.5%, respectively.

Statistical analysis. The end points of this study were death from any cause and PCa-specific death, and the survival time was calculated from the date of diagnosis to the day of death. The log-rank test was used to test for an association with survival in either the dominant, recessive, or additive model for a variant allele of each SNP, and the most suitable model with the smallest P value was adopted for each SNP. The MGF was defined as the lower frequency of 2 dichotomized genotype groups in each SNP. We used a cutoff of 0.40 for the MGF with the strategy to detect a common, high-frequency genetic abnormality.

We used a cutoff of 0.30 for the FDR to correct for the multicity problem in multiple testing. We performed a LOOCV analysis. Specifically, in each fold of LOOCV, we selected SNPs by multiple testing from scratch, developed a compound covariates prediction score for the training set, and classified the test sample into high- or low-risk groups depending on whether the test sample score was greater than the median of the prediction score in the training set.[22] After completion of the LOOCV analysis, we compared the survival time between the 2 groups for the entire sample. To assess the statistical significance of a log-rank test statistic for no survival difference, we performed a permutation method with 2,000 repetitions of the whole process of the LOOCV analysis after random permutations of the survival times.[23]

To compare the survival times according to clinicopathological factors, patients were dichotomized by the median value. The influences of SNPs and the clinicopathological factors on survival were assessed by a Cox proportional hazard regression analysis. After the LOOCV analysis, we also fit a multivariate Cox model with both the predicted risk classification based on genetic variables (i.e., the predicted high- and low-risk groups) and the clinicopathological factors as the covariates. Overall and cancer-specific survival times were estimated using the Kaplan-Meier method. SPSS software version 16.0 (SPSS Japan Inc., Tokyo, Japan) and SAS 9.2 (SAS Institute Inc., Cary, NC) were used for survival analyses, and 2-sided P values of less than 0.05 were considered to indicate a statistical significance.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Insulin-like growth factor-1 genotypes and haplotypes influence the survival of prostate cancer patients with bone metastasis at initial diagnosis

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Abstract

Background: The insulin-like growth factor-1 (IGF-1) plays an important role in growth of prostate cancer (PCa) cells and facilitating the development and progression of PCa. This study aimed to evaluate the association of polymorphisms in three linkage disequilibrium (LD) blocks of the IGF-1 on the survival of metastatic PCa patients.

Methods: A total of 215 patients with bone metastases at initial presentation were included in this study. The cytosine-adenine (*CA*) repeat polymorphism and rs12423791 were selected as representative polymorphisms in the LD blocks 1 and 2, respectively. Haplotype in the LD block 3 was analyzed using two tag single nucleotide polymorphisms (SNPs), rs6220 and rs7136446. Cancer-specific survival rate was estimated from the Kaplan-Meier curve, and the survival data were compared using the log-rank test.

Results: Cancer-specific survival was significantly associated with the *CA* repeat polymorphism, rs12423791, and rs6220 ($P = 0.013$, 0.014, and 0.014, respectively). Although rs7136446 had no significant association with survival, the haplotype in the LD block 3 was significantly associated with cancer-specific survival ($P = 0.0003$). When the sum of the risk genetic factors in each LD block (19-repeat allele, *C* allele of rs12423791, or *C-T* haplotype) was considered, patients with all the risk factors had significantly shorter cancer specific-survival than those with 0–2 risk factors ($P = 0.0003$).

Conclusions: Polymorphisms in the *IGF-1*, especially a haplotype in the LD block 3, are assumed to be genetic markers predicting the outcome of metastatic PCa.

Keywords: Prostate cancer, Bone metastasis, Survival, Polymorphism, Insulin-like growth factor-1

Background

Prostate cancer is typically a type of slow-growing cancer and generally well controlled by endocrine therapies even if distant metastases are present. However, those patients with distant metastases exhibit disease progression within 12 to 18 months on average and gradually manifest resistant to endocrine therapies thereafter [1]. Because several new promising agents are available or being tested for treatment of castration-resistant prostate cancer [2-4], it has been of importance to identify pretreatment prognostic factors in metastatic prostate cancer for adjusting treatment intensity in each patient. Clinical and laboratory factors such as extent of disease (EOD) score [5], serum alkaline phosphate (ALP) [6], hemoglobin (HGB) [7], and prostate specific antigen (PSA) [8] have been used as prognostic markers for those patients since 20 years ago. Recent studies suggest that patients' intrinsic genetic factors or an interaction with environmental factors may have an impact on progression or survival in advanced prostate cancer patients

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[9,10]. Our previous study demonstrated that the *insulin-like growth factor-1* (*IGF-1*) and the *cytochrome P450 aromatase* (*CYP19*) polymorphisms were significantly associated with the cancer -specific survival of metastatic prostate cancer [10]. However, to date, investigations of genetic polymorphisms associated with cancer progression or survival have just begun and only a few reports are available in regard to a prostate cancer [11,12]. Evaluating outcomes using genetic makers combined with conventional prognostic markers is expected to lead to more accurate prediction of response to treatments or survival.

IGF-1 is involved in embryonic growth, homeostasis, and various diseases by regulating cell differentiation, proliferation, migration, and apoptosis. In the prostate, it plays an important role in the growth of both normal and cancer cells and facilitates the development and progression of prostate cancer [13,14]. Recent meta-analysis revealed that men with higher circulating IGF-1 levels had an increased risk of prostate cancer compared with men with lower IGF-1 levels [15] and the levels of circulating IGF-1 had a heritable component [16]. The same positive association was observed between circulating IGF-1 level and a risk for breast and colorectal cancer [17-21]. Meanwhile, a cytosine-adenine (*CA*) repeat polymorphism has been known to be located in the promoter region of the *IGF-1* gene [22] and many studies investigated the influence of the polymorphism on the circulating IGF-1 level and risk for certain types of cancer [23]. Recent genome research revealed a number of single nucleotide polymorphism (SNP) throughout the *IGF-1* region and haplotype analyses demonstrated that those SNPs were divided into three to four blocks in which SNPs for each block are in linkage disequilibrium (LD) each other [24,25]. Especially a haplotype in the LD block 3 located in a downstream of the *CA* repeat has been suggested as a novel genetic variation associated with circulating IGF-1 level or cancer risk [25,26].

In this retrospective study, we aimed to evaluate the association of four polymorphisms in three LD blocks of the *IGF-1* on the survival of prostate cancer patients with bone metastasis at initial diagnosis.

Methods

Patients

From July 1980 to September 2008, 215 native Japanese patients of prostate cancer with bone metastasis at initial presentation in Akita University Hospital and its related community hospitals, Kyoto University and Tohoku University Hospitals were enrolled in this study. Pathological diagnosis was made by prostate needle biopsy specimens and metastasis was identified by X-rays, CT scans, or bone scintigraphy. All the patients had no previous treatments at presentation and underwent surgical castration or luteinizing hormone-releasing hormone (LH-RH) analogues with or without antiandrogens as primary endocrine therapy. When the treatment failure was observed, optional therapies, including other antiandrogens, estrogens, steroids, chemotherapeutic agents, palliative radiation, or a combination of these was added or replaced.

Pathological grading of needle biopsy specimens was performed according to Gleason grading system by local pathologists with no designated primary pathologist. In 10 patients, the final pathological grade was not determined because no grade information was described in the final report or a different grading system was applied by the local pathologists. Pretreatment HGB, ALP, lactate dehydrogenase (LDH), and PSA levels before the initial treatment of prostate cancer were obtained from medical charts. An independent end-point reviewer in each institution determined the cause of death on the basis of standardized extractions from the patients' medical files without providing genotype data of each patient.

Written informed consent was obtained from all the patients enrolled in this study for the use of their DNA and clinical information. This study was approved by the Institutional Review Board (the Ethical Committee) in each institution.

Genotyping analysis

DNA was extracted from a peripheral blood sample of each patient using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or standard phenol-chloroform. We divided the *IGF-1* gene into three LD blocks according to a previous report by Johansson et al. (Figure 1) [25]. The representative polymorphisms in each LD block were chosen with reference to literatures as the genes previously described to be associated with the circulating IGF-1 level or the increased risk of prostate cancer [27] and SNP database of international HapMap project (http://hapmap.ncbi.nlm.nih.gov/) [28]. The *CA* repeat polymorphism in the promoter region and rs12423791 [GenBank] were selected from representative polymorphisms in the LD block 1 and 2, respectively. rs6220 [GenBank] and rs7136446 [GenBank] were selected as tag

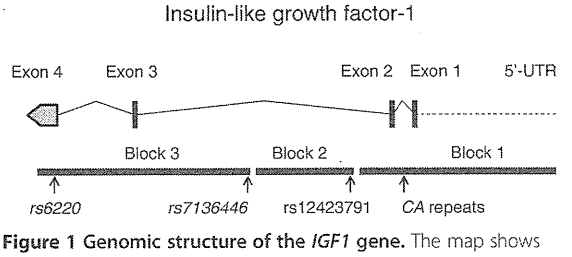

Figure 1 Genomic structure of the *IGF1* gene. The map shows the structure of the *IGF-1* gene and its linkage disequilibrium (LD) blocks. The locations of four polymorphisms analyzed in this study are indicated in the figure.

SNPs for a haplotype analysis in the LD block3. Genotypes of the *CA* repeat polymorphism were determined by an automated sequencer (ABI PRISM 310 Genetic Analyzer) with GENESCAN software (Applied Biosystems, Foster City, CA) as described previously [17]. Other three SNPs were genotyped using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. The sequence of forward and reverse primers used for genotyping of the SNPs were as follows; 5′-GCTGC TTCTTCCAATGAGAG-3′ and 5′-GAAAAGCATGTTGC TGCCTC-3′ for rs12423791 (123 bp), and TGCCTAG AAAAGAAGGAATC-3′ and 5′-TGACTCTTCTATGCAG TTAC-3′ for rs6220 (105 bp), and 5′-CTTCTTGCAGAAC TAAGCTCAAGTC-3′ and 5′-GCCTATTCATTTTCA CATACTACCC-3′ for rs7136446 (126 bp). Each PCR product was digested with DdeI, MspI, and MnlI, respectively, overnight at 37°C, and electrophoresed on 3.0% agarose gels to determine the genotype. Several samples were directly sequenced using Dye Terminator Sequencing Kit version 1.0 (PE Applied Biosystems) on an ABI prism 310 auto-sequencer to confirm the results of PCR-RFLP for each polymorphism.

Statistical analysis

The endpoint of this study was prostate cancer-specific survival. The survival time was calculated from the date of prostate cancer diagnosis to the date of death or the last contact with patients. To compare the survival, patients were dichotomized by the median value of age and PSA, by normal limits in HGB, ALP, LDH, by the tumor grade system (Gleason score). Haplotypes of the LD block 3 defined two SNPs, rs6220 and rs7136446, were inferred using expectation-maximization algorithm in SNPAlyze software ver.7 (Dynacom Co. Ltd., Chiba, Japan). Differences in survival between groups were analyzed using the logrank test. For the *CA* repeat polymorphism, the number of repeats was dichotomized as having or not having the 19-repeat allele. Each SNP or haplotype was evaluated using dominant, recessive, and addictive model, and the most statistically significant model was selected. The *IGF-1* polymorphisms and clinicopathological prognostic factors were assessed by the Cox proportional hazard regression models. Age, Gleason score, PSA, HGB, ALP, LDH, and the LD block 3 haplotype were employed as a variable set in a full-variable model of multivariate analysis. Of the variables, Gleason score, HGB, ALP, and the LD block 3 haplotype were selected in a reduced variable model. Cancer-specific survival was estimated using the Kaplan–Meier method. All the statistical analyses were performed using SPSS software version 19.0 (IBM Japan Ltd., Tokyo, Japan) and two-sided *P* values of less than 0.05 were considered to indicate statistical significance.

Results

Clinicopathological background of patients

The mean age (± SD) of the 215 patients was 70.2 ± 8.4 - years (range, 45–89; median, 72 years). The mean follow-up period was 46.4 ± 36.1 months (range, 1–209; median, 37 months). Of 215 patients, bone metastasis alone, additional lymph nodes metastasis, and other visceral metastasis were seen at initial diagnosis in 106 (49.3%), 98 (45.6%), and 15 (7.0%), respectively. The distribution of Gleason score of biopsy specimen was < 7 in 15 patients (7.0%), 7–8 in 81 (37.7%), 9–10 in 109 (50.7%), and unknown in 10 (4.6%). Pretreatment PSA, HGB, ALP, and LDH levels are shown in Table 1. All the patients were received an endocrine therapy as an initial treatment, surgical castration alone in 29 (13.5%), LH-RH analogue alone in 53 (24.6%), combined androgen blockade in 131 (60.9%). Among 188 patients with available data, 91patinets (48.4%) achieved a PSA nadir less than 1 ng/ml, while 97 patients (51.6%) did not reach the level after initial endocrine therapies.

Genotyping analysis

The repeat number of the *CA* repeat polymorphism ranged from 13 to 20, and 20 genotypes were observed. The distributions of the genotypes of the *CA* repeat polymorphisms, rs12423791, rs6220, and rs7136446 were shown in Table 2. Estimated haplotype frequencies of the LD block 3 (rs6220 - rs7136446) were 55.2% (*T-T*), 26.7% (*C-T*), 16.8% (*C-C*), and 1.3% (*T-C*). Thirty-seven patients with heterozygous genotype of both rs6220 and rs7136446 were estimated as having the *T-T* and *C-C* haplotypes because estimated haplotype frequency of the *T-C* haplotype

Table 1 Patients' clinical characteristics

	Mean ± SD (median)	Range
Age (years)	70.2 ± 8.4 (72)	45 – 89
PSA (ng/mL)	1,029 ± 1,896 (260)	2.4 – 12,490
HGB (g/dL)	13.1 ± 1.9 (13.3)	7.4 – 17.4
ALP (IU/L)	615 ± 908 (291)	50 – 5,870
LDH (IU/L)	295 ± 180 (222)	97 – 1,273
Follow-up (months)	46.4 ± 36.1 (37)	1 – 209
	N (%)	
Metastases		
Bone only	106 (49.3)	
Lymph nodes	98 (45.6)	
Other organs	15 (7.0)	
Gleason score		
< 7	15 (7.0)	
7 – 8	81 (37.7)	
9 – 10	109 (50.7)	
Unknown	10 (4.6)	

Table 2 Genotype distributions of four polymorphisms analyzed in this study

Genotype	N (%)
CA repeat	
13/15	1 (0.5)
14/17	1 (0.5)
15/16	4 (1.9)
15/17	11 (5.1)
15/18	3 (1.4)
15/19	12 (5.6)
15/20	1 (0.5)
16/16	2 (0.9)
16/17	25 (11.6)
16/18	7 (3.3)
16/19	17 (7.9)
17/17	16 (7.4)
17/18	18 (8.4)
17/19	48 (22.3)
17/20	1 (0.5)
18/18	9 (4.2)
18/19	16 (7.4)
18/20	1 (0.5)
19/19	15 (0.5)
19/20	7 (7.0)
rs12423791	
GG	140 (65.1)
GC	75 (34.9)
rs6220	
TT	71 (33.0)
TC	102 (47.5)
CC	42 (19.5)
rs7136446	
TT	146 (67.9)
TC	60 (27.9)
CC	9 (4.2)

was quite low. Haplotypes in all other patients were uniquely determined.

Survival analysis

Kaplan-Meier curves demonstrated that patients with 19-repeat allele, *C* allele of rs12423791, or *C* allele of rs6220 had a significantly worse survival ($P = 0.013$, 0.014, or 0.014, respectively). Whereas, rs7136446 was not associated with patients' survival ($P = 0.371$). Patients with at least one *C-T* haplotype showed significantly worse survival compared with those who had no *C-T* haplotype ($P = 0.0003$) (Figure 2). When the number of the genetic risk factors (19-repeat allele, *C* allele of rs12423791, or *C-T* haplotype) was considered, cancer-specific survival significantly shortened with increased the number of genetic risk factors ($P = 0.002$), and patients with all the genetic risk factors had significantly shorter survival than those with 0–2 risk factors ($P = 0.0003$) (Figure 3).

An univariate Cox proportional hazard regression analysis showed that cancer-specific survival was significantly lower in patients with Gleason score of 9 or higher (HR: 1.759, 95% CI: 1.151-2.687, $P = 0.009$), HGB less than 11.5 g/dl (HR: 2.251, 95% CI: 1.261-4.019, $P = 0.006$), ALP of 350 IU/ml or higher (HR: 2.836, 95% CI: 1.756-4.578, $P = 0.00002$), or LDH of 500 IU/ml or higher (HR: 2.638, 95% CI: 1.442-4.829, $P = 0.002$) (Table 3). Meanwhile, neither dichotomized age nor PSA was associated with cancer-specific survival. In a multivariate analysis including all the clinicopathological variables and haplotype of the LD block 3 as a representative genetic variable, higher Gleason score (HR: 1.766, 95% CI: 1.052-2.966, $P = 0.031$), higher ALP (HR: 2.598, 95% CI: 1.483-4.551, $P = 0.0008$), and the *C-T* haplotype (HR: 2.619, 95% CI: 1.559-4.399, $P = 0.0003$) were independent factors predicting cancer-specific survival. HGB and LDH showed borderline significance ($P = 0.061$ and 0.059, respectively). In a reduced variable model, higher ALP (HR: 2.819, 95% CI: 1.695-4.689, $P = 0.00007$) and *C-T* haplotype (HR: 2.626, 95% CI: 1.603-4.305, $P = 0.0001$) were stronger independent predictors for the survival, followed by HGB (HR: 2.082, 95% CI 1.113-3.897, $P = 0.022$) and Gleason score (HR: 1.709, 95% CI: 1.054-2.771, $P = 0.030$) (Table 3).

Because the Gleason score and the pretreatment ALP level were shown to be significant prognostic factors along with the LD Block 3 haplotype by multivariate analysis (Table 3), we performed subgroup analyses according to the dichotomized Gleason score or the dichotomized pretreatment ALP level to compare survivals by presence or absence of *C-T* haplotype. Among patients with Gleason score of 9–10 (n = 108), those with C-T haplotype showed significantly worse survival than those having no *C-T* haplotype ($P = 0.0002$), while there was no significant difference ($P = 0.365$) in patients with Gleason score less than 9 (n = 96). Regarding ALP, patients with *C-T* haplotype showed significantly shorter survival than those with no *C-T* haplotype in either subgroup (ALP higher or lower than 350 IU/ml) ($P = 0.010$ or 0.009, respectively) (Figure 4).

Discussion

The association of the *CA* repeat polymorphism in the promoter region of the *IGF-1* with circulating IGF-1 levels and a risk of breast, prostate, and colorectal cancers have been extensively evaluated [23,29]. In those previous studies, the *CA* repeat polymorphisms were

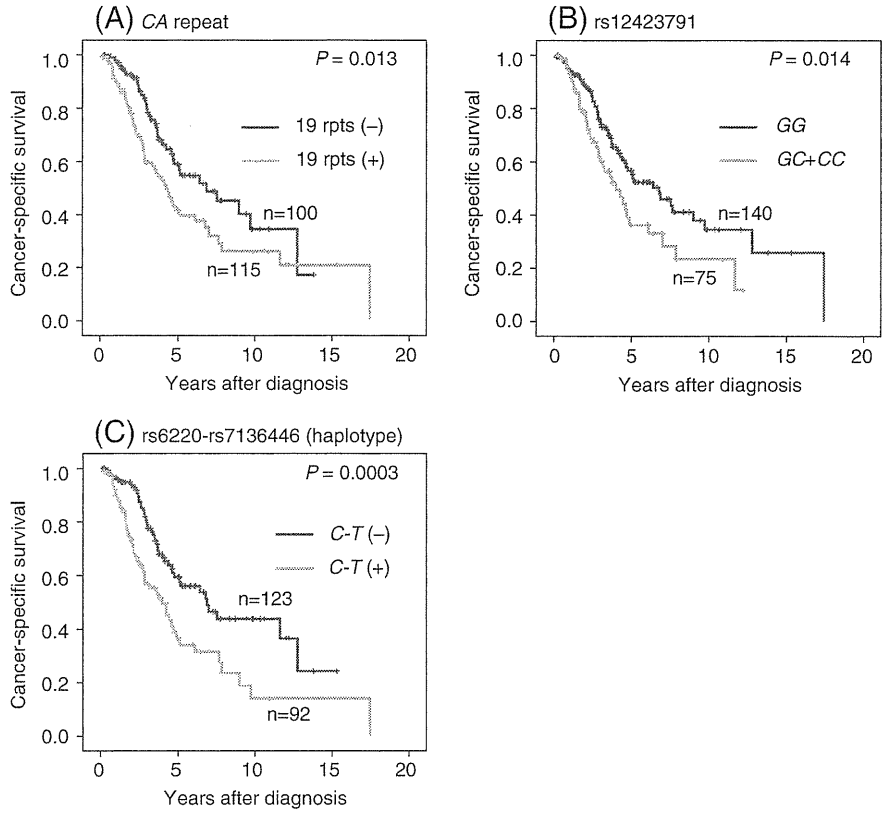

Figure 2 Cancer-specific survival of patients classified based on the risk allele, genotype or haplotype. The survival was significantly worse in patients with 19 *CA* repeat allele in the LD block1 than those without 19 *CA* repeat allele ($P = 0.013$) (**A**). Patients with *GC* or *CC* genotype of the rs12423791 in the LD block 2 had significantly worse survival than those with *GG* genotype ($P = 0.014$) (**B**). As regards to the LD block 3 haplotype, patients with *C-T* haplotype had significantly worse survival than those without *C-T* haplotype ($P = 0.0003$) (**C**).

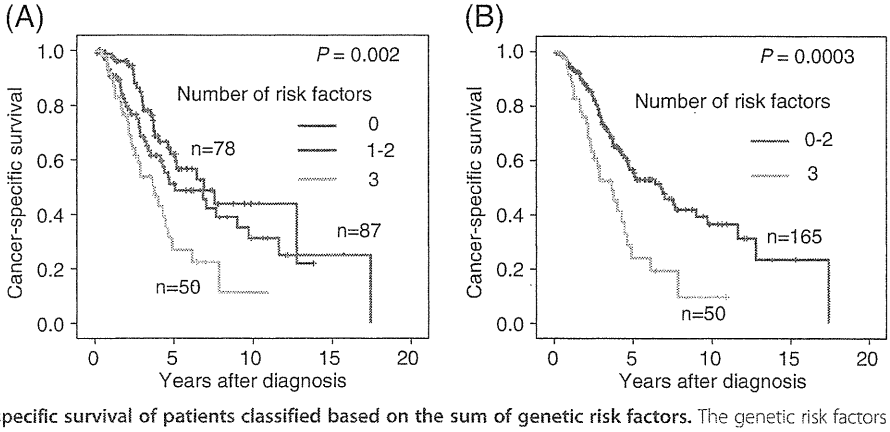

Figure 3 Cancer-specific survival of patients classified based on the sum of genetic risk factors. The genetic risk factors were defined as presence of 19 repeat allele of the *CA* repeat polymorphism, *C* allele of the rs12423791, and *C-T* haplotype of the LD block 3. Each patient was assigned to one of 3 groups (0, 1–2, or 3 risk factors) (**A**) or one of 2 groups (0–2 or 3 risk factors) (**B**). The cancer-specific survivals significantly differed in both of the classification ($P = 0.002$ and 0.0003).

Table 3 Univariate and multivariate analysis of clinicopathological and genetic factors predicting cancer-specific survival

	Category	HR[1]	95% CI[2]	*P*
Univariate analysis				
Clinical and pathological factors				
Age (yrs)	≥ 72 vs. < 72	1.183	0.790 - 1.772	0.415
Gleason score	≥ 9 vs. < 9	1.759	1.151 – 2.687	0.009
PSA (ng/mL)	≥ 260 vs. < 260	1.510	0.992 – 2.299	0.054
HGB (g/dL)	< 11.5 vs. ≥ 11.5	2.251	1.261 – 4.019	0.006
ALP (IU/L)	≥ 350 vs. < 350	2.836	1.756 – 4.578	0.00002
LDH (IU/L)	≥ 500 vs. < 500	2.638	1.442 – 4.829	0.002
Genetic factors				
LD block 1 (*CA* repeat)	19 rpts (+) vs. (–)	1.671	1.109 – 2.518	0.014
LD block 2 (rs12423791)	*GC* + *CC* vs. *GG*	1.658	1.102 – 2.495	0.015
LD block 3 (haplotype)	*C-T* (+) vs. *C-T* (–)	2.054	1.373 – 3.075	0.0005
Number of risk factors	0 vs. 1–2 vs. 3	1.578	1.208 – 2.060	0.0008
	0–2 vs. 3	2.202	1.414 – 3.430	0.0005
Multivariate analysis				
Full model				
Age (yrs)	≥ 72 vs. < 72	0.919	0.554 – 1.526	0.745
Gleason score	≥ 9 vs. < 9	1.766	1.052 – 2.966	0.031
PSA (ng/mL)	≥ 265 vs. < 265	0.932	0.496 – 1.749	0.826
HGB (g/dL)	< 11.5 vs. ≥ 11.5	2.012	0.968 – 4.180	0.061
ALP (IU/L)	≥ 350 vs. < 350	2.598	1.483 – 4.551	0.0008
LDH (IU/L)	≥ 500 vs. < 500	1.836	0.977 – 3.448	0.059
LD Block 3 (haplotype)	*C-T* (+) vs. *C-T* (–)	2.619	1.559 – 4.399	0.0003
Reduced model				
Gleason score	≥ 9 vs. < 9	1.709	1.054 – 2.771	0.030
HGB (g/dL)	< 11.5 vs. ≥ 11.5	2.082	1.113 – 3.897	0.022
ALP (IU/L)	≥ 350 vs. < 350	2.819	1.695 – 4.689	0.00007
LD Block 3 (haplotype)	*C-T* (+) vs. *C-T* (–)	2.626	1.603 – 4.305	0.0001

[1]HR, hazard ratio; [2]95% CI, 95% confidence interval.

generally categorized as having a 19-repeat allele or not having the allele. To date, however, the results were inconsistent in terms of whether 19-repeat allele increases IGF-1 levels or cancer risks. A recent large-scale study of 6,400 healthy subjects indicated that other polymorphisms downstream of the *CA* repeat polymorphism may affect IGF-1 circulation levels [30]. In prostate cancer patients, Johansson et al. demonstrated that heterozygous haplotype of *T-C-C* (rs6220-rs7136446-rs2033178 [GenBank]) in the 3′ region of the *IGF-1*, which was a risk haplotype of prostate cancer risk in their previous study, was significantly associated with higher circulating levels of IGF-1 [27]. In the study, however, another cohort did not show the significant difference in circulating IGF-1 levels and rather patients with rs6220 *CC* genotype showed significantly higher circulating IGF-1 level in a separate SNP analysis of each SNP [27]. Other study also showed significantly increased circulating IGF-1 levels in females with rs6220 *CC* genotype [31]. Since all the patients in our series had the *CC* genotype at rs2033178 (data not shown), the *T-C-C* haplotype is referred to as the *T-C* haplotype in our study. Although the *T-C* (*T-C-C*) haplotype was not separately analyzed in a survival analysis due to the rare haplotype with only 1.3%, our result showed that patients with the *C-T* haplotype had significantly worse survival and appeared to be in line with previous studies investigating the association between circulating IGF-1 levels and the *IGF-1* polymorphisms.

Multiple interpretations are possible regarding the role of the polymorphisms in altering the circulating IGF-1 levels. A previous study demonstrated in other genes

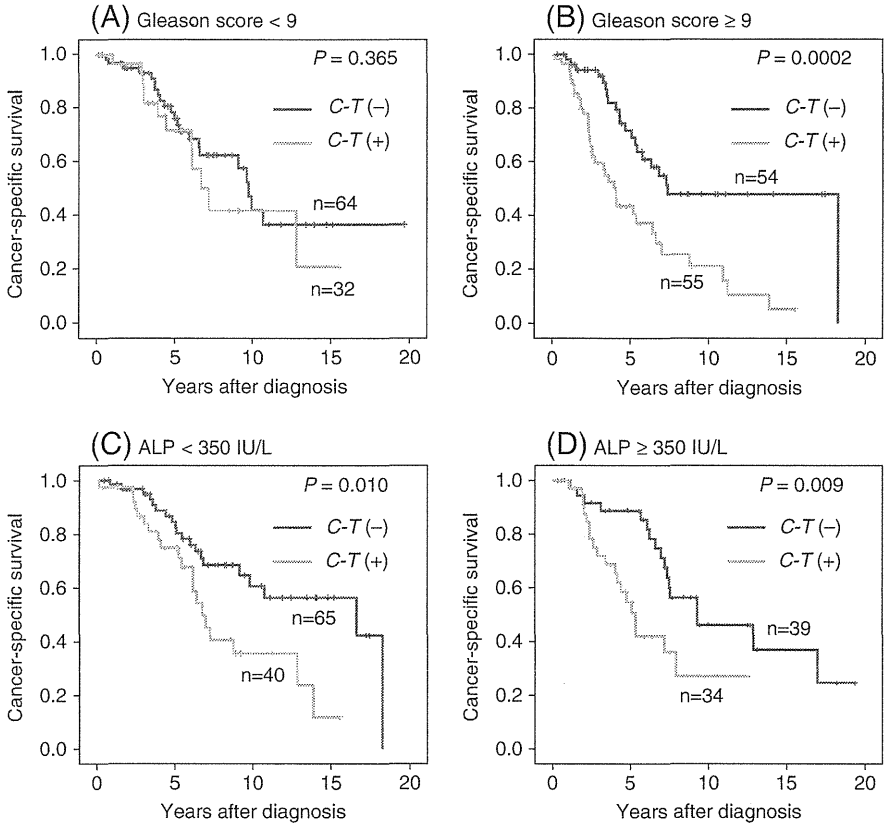

Figure 4 Cancer-specific survival of patients classified based on haplotype in the LD block 3 in subgroups dichotomized by Gleason score or pretreatment ALP level. Patients with *C-T* haplotype showed significantly worse survival than those having no *C-T* haplotype ($P = 0.0002$) in patients with Gleason score of 9–10 (**A**), while there was no significant difference ($P = 0.365$) in patients with Gleason score less than 9 (**B**). In a subgroup analysis by pretreatment ALP level, patients with *C-T* haplotype showed significantly shorter survival than those with no *C-T* haplotype in either subgroup of patients with lower (< 350 IU/L) or higher (≥ 350 IU/L) ALP ($P = 0.010$ or 0.009, respectively) (**C** and **D**).

that the *CA* repeat in the promoter region acted as a negative control element [32], suggesting a possibility that a *CA* repeat length directly affects the transcriptional activity of the *IGF-1*. This hypothesis is partially supported by a study conducted by Missmer et al. [33]. They showed a trend of decreasing IGF-1 level with increasing the *CA* repeat length genotype, although this was not statistically significant [33]. Another explanation is that *CA* repeats do not directly affect the transcriptional activity but other SNPs being in linkage disequilibrium exert functional effect on a transcriptional activity. Recent studies demonstrated that SNPs or haplotypes in other regions in the *IGF-1*, especially downstream of the *CA* repeats, were associated with circulating IGF-1 levels or cancer susceptibility [24,30]. Chen et al. reported a possible association of a haplotype combined of SNPs and the *CA* repeat length with circulation IGF-1 levels. In the study, the combined haplotype was correlated with circulating IGF-1 levels and neither SNPs nor the *CA* repeat alone was associated with the IGF-1 levels [34]. Nevertheless, in vitro studies are needed to determine the functional implication of those genetic polymorphisms on the alteration of IGF-1 expression.

Several mechanisms of IGF-1 affecting the prognosis of metastatic prostate cancer are envisioned. First, IGF-1 is known to act as an important growth factor regulating proliferation and apoptosis of cancer cells and has a role in an acquisition of resistance to endocrine therapies [35-37]. Secondary, IGF-1, which is also produced by bone cells, down-regulates osteoprotegerin (OPG) and up-regulated receptor activator of NF-κB ligand (RANKL) [38]. Results of the present study suggest that the polymorphisms are associated with an aggressive phenotype and resistance to endocrine therapy and facilitate the progression of prostate cancer cells especially in bone metastasis.

The present study has several limitations. First, the present study has a possible bias of patient selection, which is a drawback of retrospective study design. Although the majority of the patients are incident cases, some patients who had rapid progression and very short-

term survival may have not been enrolled into the study. Second, treatment strategy was not regulated in this retrospective study. Because of the long recruiting period, various treatments except for endocrine therapies including docetaxel were administered only in recent cases. A prospective study with a large cohort is mandatory to validate the results. Thirdly, we examined only 4 polymorphism loci and other SNPs may have a stronger association with the survival than those evaluated in the present study. A study using precise SNP panel may lead to identify SNPs truly responsible for the survival and the function of the SNPs should be supported by biological investigations.

Conclusions

Polymorphisms of the *IGF-1*, especially *C-T* haplotype in the LD block 3 were associated with worse survival of prostate cancer patients with bone metastasis at initial diagnosis. The genomic variations in the *IGF-1* combined with conventional clinicopathological prognostic markers, along with conventional clinical markers, appeared to be useful for predicting the outcome of metastatic prostate cancer.

Abbreviations

EOD: Extent of disease; ALP: Alkaline phosphate; HGB: Hemoglobin; PSA: Prostate specific antigen; IGF-1: Insulin-like growth factor-1; CYP19: Cytochrome P450 aromatase; SNP: Single nucleotide polymorphism; LH-RH: Luteinizing hormone-releasing hormone; LDH: Lactate dehydrogenase; PCR: Polymerase chain reaction; RFLP: Restriction fragment length polymorphism; RANKL: Receptor activator of NF-κB ligand.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NT and TH were involved in the conception and design of the study. SN, TI, and MH performed laboratory work. MS, KN, SS, SH, and OO were involved in the provision of study material and patients' clinical data. NT and TH drafted the manuscript. SS, CO, YA, OO supported the manuscript writing. All authors have read and approved the final manuscript.

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