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Laboratory-Prostate cancer

Genetic polymorphism and carbonic anhydrase 9 expression can predict nodal metastatic prostate cancer risk in patients with prostate-specific antigen levels ≤10 ng/ml at initial biopsy

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Abstract

Objectives: Active surveillance is a common management method for low-risk prostate cancer (CaP). However, devising a method to prevent disease progression is crucial. Carbon anhydrase 9 (CA9) plays a vital role in cell adhesion and intercellular communication correlated to tumor metastasis. Our study explored the impact of CA9 genetic polymorphism on the clinicopathological features and prognosis of CaP.

Materials and methods: In total, 579 patients with CaP who underwent robot-assisted radical prostatectomy were enrolled, 270 of whom had an initial prostate-specific antigen (PSA) level ≤10 ng/ml and 309 had initial one >10 ng/ml. Three single-nucleotide polymorphisms of CA9 gene were examined using real-time polymerase chain reaction assay.

Results: After adjusting the confounding factors, participants carrying at least one G allele at CA9 rs3829078 had a 2.241-fold change in PSA compared with the wild-type carrier (AA), leading to an initial PSA level of ≤10 ng/ml. Furthermore, patients with CaP with an initial PSA level ≤10 ng/ml who carried at least one G allele at CA9 rs3829078 had a 4.532-fold and 3.484-fold risk of lymph node metastasis and lymphovascular invasion, respectively. Moreover, The Cancer Genome Atlas database showed that the CA9 mRNA expression significantly increased N1 disease risk and worsened overall survival trends.

Conclusion: The rs3829078 polymorphic genotype of CA9 can predict the risk of lymph node metastasis of CaP with an initial PSA level ≤10 ng/ml. This is the first study to report a correlation between CA9 gene polymorphisms/CA9 mRNA expression and early detection of CaP.

Keywords: Carbonic anhydrase 9; Polymorphism; Prostate cancer; Prostate-specific antigen

1. Introduction

Prostate cancer (CaP) is one of the most common cancers, and it is also one of the major reasons for cancer deaths among men. In the United States alone, approximately 164,690 patients are newly diagnosed with CaP, and approximately 29,430 men will die from this disease in 2018 [1]. More than 1 million prostate biopsies that were performed annually for disease detection were owing to increasing or fluctuating levels of prostate-specific antigen (PSA) [2]. The extensive use of PSA testing has caused a

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drastic increase in CaP detection and treatment. However, low specificity of the PSA level in CaP detection and relatively poor prediction of tumor aggressiveness have led to an increase in unnecessary biopsies and a diagnosis of indolent CaPs, especially in men with a PSA level <10 ng/ml, who have only a 22% to 27% likelihood of cancer development [3,4]. The overdiagnosis and overtreatment of low-risk CaP can cause adverse effects and are harmful for these patients [5]. Moreover, in men with low- to intermediate-risk CaP, the overall survival was reported to be 10 years with no difference between active monitoring and active treatment [6]. Therefore, active surveillance (AS) is an acceptable choice of primary treatment for very low-, low-, and selected intermediate-risk CaPs. However, 3% to 6% patients have developed a metastatic disease during AS [6,7]. Determining the risk factor for clinical progression and metastasis development is crucial.

Carbon anhydrases (CAs), a family of zinc metalloenzymes, catalyzed carbon dioxide hydration and produced protons and bicarbonate. At least 13 human CA isozymes collaborate to control intracellular and extracellular acid-base balance [8]. Carbon anhydrase 9 (CA9) is a transmembrane enzyme with an extracellular catalytic domain, which leads to acidification of the outer microenvironment [9]. CA9 expression plays a crucial role in cell adhesion and intercellular communication that correlates with a high metastasis risk and poor prognosis in several human tumors [10–13]. Although the correlation between CA9 expression and tumor grading of CaP cells has been controversial, [14,15] the activity of serum CA9 has been proven to play a key role in CaP cell growth and poor prognostic factors for survival in castration-resistant prostate cancer, [13,16] Moreover, in an in vivo study, stromal CA9 concentration had an upstream hierarchical role in the metastasis of prostate carcinoma cell [17].

Single-nucleotide polymorphism (SNP) occurs when a nucleotide from the shared sequence of a gene varies by >1% within a population. One study showed that SNPs in the exon region of CA9 gene can predict overall survival of metastatic renal cell carcinoma [18]. Several genetic polymorphisms have been associated with the PSA level, CaP risk, tumor grading, and CaP-specific mortality [19–21]. However, no studies have focused on the correlation between CA9 polymorphism and metastatic CaP risk with a PSA level ≤10 ng/ml at initial diagnosis. To our knowledge, this is the first study to demonstrate a significant association between CA9 gene polymorphism and metastatic CaP risk.

2. Materials and methods

2.1. Description of enrolled subjects

We recruited 579 patients with adenocarcinoma of the prostate who underwent robot-assisted radical prostatectomy with bilateral standard pelvic lymph node dissection during 2012 to 2017 at Taichung Veteran General Hospital. Before the initiation of this study, the protocol was approved by the Institutional Review Broad (IRB) of Taichung Veteran General Hospital, and informed written consent was obtained from each participant (IRB No. CG12139). Medical information was acquired from patient medical records, which included the initial PSA at diagnosis, clinical and pathological tumor-node-metastasis (TNM) staging, Gleason score of initial biopsy, D'Amico classification, [22] Gleason grade group, and other pathological features of the permanent pathological report [23]. Patients with CaP were staged according to the TNM staging system of the eighth edition of the American Joint Committee on Cancer Staging Manual. We divided the patients into 2 groups, namely 270 patients with initial PSA level ≤10 ng/ml (iPSA ≤ 10 group) and 307 patients with initial PSA level >10 ng/ml (iPSA > 10 group).

2.2. Specimen collection and genomic DNA extraction

We collected 579 blood samples from patients with CaP before surgery. The whole-blood specimens were placed in tubes with ethylenediaminetetraacetic acid, centrifuged immediately, and stored at −80°C. Genomic DNA was extracted with QIAamp DNA blood mini kits (Qiagen, Valencia, CA, USA) based on the manufacturer's protocol [24].

2.3. Selection of CA9 genetic polymorphism

According to the National Center for Biotechnology Information database, >30 genetic polymorphisms exist over the exon region of CA9 gene. We selected rs2071676 (+201, G/A) in exon 1, rs3829078 (+1081, A/G) in exon 7, and rs1048638 (+1584, C/A) in 3′-UTR of exon 11 based on the potential involvement of various cancers [18,25–29]. The genotypic frequencies of these 3 CA9 SNPs were assessed using a StepOne Real-time PCR System (Applied Biosystems, Foster City, CA, USA) and analyzed using the TaqMan assay with SDS 3.0 software (Applied Biosystems). The details of methods, probes, and primer sequences for determining CA9 gene polymorphism were reported in our previous study [25].

2.4. Statistical analysis

Chi-square and Student's *t* test were used to compare the demographic characteristics between the 2 groups, namely PSA ≤ 10 and > 10, at diagnosis. The odds ratios (ORs) and adjusted ORs (AORs) with 95% confidence intervals (CIs) were estimated using multiple logistic regression models to determine the association between genotypic frequencies and the 2 PSA groups and the risk of different clinicopathological characteristics. We analyzed our data using SAS version 9.1 for Windows (SAS Institute,

Cary, NC, USA). A significant difference was defined as $P < 0.05$.

3. Results

3.1. Characteristics of the study participants

The demographic characteristics of the 2 PSA groups (270 patients with the PSA level ≤10 ng/ml and 307 patients with the PSA level >10 ng/ml) were analyzed (Table 1). Compared with the iPSA > 10 group, the iPSA ≤ 10 group had significantly more patients aged ≤65 years, pathological Gleason grade group 1 + 2 + 3, clinical T1 + 2, and pathologic T2, and less patients with clinical M1 disease, pathologic N1, seminal vesical invasion, perineural invasion, and lymphovascular invasion. The pathology proved that lymph node metastasis diseases in the iPSA ≤ 10 and iPSA > 10 groups were 4.1% and 12.3%, respectively. According to D'Amico classification, the percentages of low-, intermediate-, and high-risk CaP were 0% (0), 29.1% (90), and 70.1% (219) in the iPSA ≤ 10 group and 22.2% (60), 48.1% (130), and 29.7% (80) in the iPSA >10 group, respectively.

Table 1
The distributions of demographical characteristics in 579 patients with prostate cancer

Variable	PSA at diagnosis (ng/ml)		
	>10 (n = 309)	≤10 (n = 270)	P value
Age at diagnosis (y)			
≤65	113 (36.6 %)	132 (48.9 %)	0.003*
>65	196 (63.4 %)	138 (51.1 %)	
Pathologic Gleason grade group			
1 + 2 + 3	234 (75.7 %)	250 (92.6 %)	<0.001*
4 + 5	75 (24.3 %)	20 (7.4 %)	
Clinical T stage			
1 + 2	248 (80.3 %)	253 (93.7 %)	<0.001*
3 + 4	61 (19.7 %)	17 (6.3 %)	
Clinical N stage			
N0	299 (96.8 %)	263 (98.9 %)	0.085
N1	10 (3.2 %)	3 (1.1 %)	
Clinical M stage			
M0	299 (96.8 %)	270 (100.0 %)	0.003*
M1	10 (3.2 %)	0 (0.0 %)	
Pathologic T stage			
2	120 (38.8 %)	186 (68.9 %)	<0.001*
3 + 4	189 (61.2 %)	84 (31.1 %)	
Pathologic N stage			
N0	271 (87.7 %)	259 (95.9 %)	<0.001*
N1	38 (12.3 %)	11 (4.1 %)	
Seminal vesicle invasion			
No	208 (67.3 %)	244 (90.4 %)	<0.001*
Yes	101 (32.7 %)	26 (9.6 %)	
Perineural invasion			
No	62 (20.1 %)	93 (34.4%)	<0.001*
Yes	247 (79.9 %)	177 (65.6 %)	
Lymphovascular invasion			
No	236 (76.4 %)	246 (91.1 %)	<0.001*
Yes	73 (23.6 %)	24 (8.9 %)	
D'Amico classification			
Low risk	0 (0.0 %)	60 (22.2 %)	<0.001*
Intermediate risk	90 (29.1 %)	130 (48.1 %)	
High risk	219 (70.9 %)	80 (29.7 %)	

3.2. Association of CA9 gene polymorphisms and CaP with different PSA levels

Table 2 presents the distribution frequency of *CA9* genotypes in 579 patients with CaP in the different groups. AORs with 95% CIs were estimated using multiple logistical regression models after controlling for age at diagnosis, pathologic Gleason score group, clinical T stage, clinical M stage, pathologic T stage, pathologic N stage, seminal vesicle invasion, perineural invasion, lymphovascular invasion, and D'Amico classification to eliminate possible interferences.

The percentage of patients carrying the A/G genotype of the rs3829078 polymorphism is significantly higher in the iPSA ≤ 10 group (AOR = 2.209, 95% CI = 1.102−4.428, P = 0.025). Compared with the A/A genotype of rs3829078, the A/G + G/G genotype demonstrated a higher percentage in the iPSA ≤10 group (AOR = 2.241, 95% CI = 1.121−4.480, P = 0.022). No other significant difference was noted in the other 2 loci, namely rs2071676 and rs1048638.

3.3. Correlation of CA9 SNPs and clinical status of CaP with initial PSA ≤ 10 ng/ml

Because the genotype polymorphism of rs3829078 appeared to be correlated to CaP with initial PSA levels ≤10 ng/ml, we further analyzed the association of clinicopathological characteristics in the iPSA ≤10 group, as shown in Table 3. In this group, compared with patients carrying the A/A genotype, those carrying the A/G + G/G genotype showed a higher incidence of lymphovascular invasion in pathological feature (OR = 3.484, 95% CI = 1.321−9.187, P = 0.008) and pathological N1 disease (OR = 4.532, 95% CI = 1.250−16.427, P = 0.013); however, no differences were observed in other well-known prognostic factors, including clinical/pathological T stage, Gleason grade group, and distribution of D'Amico classifications.

Moreover, the CA9 mRNA level, PSA level at diagnosis, pathological T/N stage, and overall survival were assessed from The Cancer Genome Atlas (TCGA) database. In TCGA, no statistical difference was observed between the T2 and T3 + 4 groups of CaP in terms of CA9 mRNA expression (Fig. 1A), but a trend of high CA9 mRNA expression was observed in pathological N1 disease. However, the difference was statistically nonsignificant (P = 0.0558, Fig. 1B). Subsequently, we focused on patients with initial PSA levels ≤10 ng/ml. No statistical differences were noted among various pathological T stages in terms of CA9 mRNA expression (P = 0.0908, Fig. 1C). However, the CA9 mRNA level was significantly higher in the patho-

Table 2
Distribution frequency of *CA9* genotypes in 579 patients with prostate cancer

Variable	PSA at diagnosis (ng/ml)			
	>10	≤10	OR (95% CI)	AOR (95% CI)
rs2071676				
AA	81 (26.2%)	74 (27.4%)	1.00	1.00
AG	152 (49.2%)	134 (49.6%)	0.965 (0.652−1.427)	0.845 (0.539−1.325)
GG	76 (24.6%)	62 (23.0%)	0.893 (0.564−1.415)	0.861 (0.507−1.461)
AG + GG	228 (73.8%)	196 (72.6%)	0.941 (0.651−1.360)	0.850 (0.557−1.297)
rs3829078				
AA	290 (93.9%)	237 (87.8%)	1.00	1.00
AG	18 (5.8%)	32 (11.9%)	2.175 (1.191−3.973) $P = 0.011$*	2.209 (1.102−4.428) $P = 0.025$*
GG	1 (0.3%)	1 (0.3%)	1.224 (0.076−19.667)	−
AG + GG	19 (6.1%)	33 (12.2%)	2.125 (1.178−3.834) $P = 0.011$*	2.241 (1.121−4.480) $P = 0.022$*
rs1048638				
CC	269 (87.1%)	237 (87.8%)	1.00	1.00
CA	39 (12.6%)	32 (11.9%)	0.931 (0.565−1.534)	0.959 (0.542−1.697)
AA	1 (0.3%)	1 (0.3%)	1.135 (0.071−18.246)	0.409 (0.025−6.761)
CA + AA	40 (12.9%)	33 (12.2%)	0.936 (0.572−1.533)	0.932 (0.531−1.636)

The odds ratio and with their 95% confidence intervals were estimated by logistic regression models. The adjusted odds ratios with their 95% confidence intervals were estimated by multiple logistic regression models after controlling for Age at diagnosis, pathologic Gleason grade group, clinical T stage, clinical M stage, pathologic T stage, pathologic N stage, seminal vesicle invasion, perineural invasion, lymphovascular invasion, and D'Amico classification.
* $P < 0.05$ as statistically significant.

Table 3
Odds ratio and 95% confidence interval of clinical status and *CA9* rs3829078 genotypic frequencies in 270 patients with prostate cancer with PSA concentration under 10 ng/ml

Variable	Genotypic frequencies			
rs3829078	AA ($N = 237$)	AG + GG ($N = 33$)	OR (95% CI)	P value
Pathologic Gleason grade group				
1 + 2 + 3	221 (93.2%)	29 (87.9%)	1.00	0.270
4 + 5	16 (6.8%)	4 (12.1%)	1.905 (0.596−6.090)	
Clinical T stage				
1 + 2	224 (94.5%)	29 (87.9%)	1.00	0.141
3 + 4	13 (5.5%)	4 (12.1%)	2.377 (0.726−7.777)	
Pathologic T stage				
2	162 (68.4%)	24 (72.7%)	1.00	0.611
3 + 4	75 (31.6%)	9 (27.3%)	0.810 (0.359−1.827)	
Pathologic N stage				
N0	230 (97.0%)	29 (87.9%)	1.00	0.013*
N1	7 (3.0%)	4 (12.1%)	4.532 (1.250−16.427)	
Seminal vesicle invasion				
No	217 (91.6%)	27 (81.8%)	1.00	0.075
Yes	20 (8.4%)	6 (18.2%)	2.411 (0.890−6.529)	
Perineural invasion				
No	83 (35.0%)	10 (30.3%)	1.00	0.593
Yes	154 (65.0%)	23 (69.7%)	1.240 (0.563−2.728)	
Lymphovascular invasion				
No	220 (92.8%)	26 (78.8%)	1.00	0.008*
Yes	17 (7.2%)	7 (21.2%)	3.484 (1.321−9.187)	
D'Amico classification				
Low risk/Intermediate risk	166 (70.0%)	24 (72.7%)	1.00	0.752
High risk	71 (30.0%)	9 (27.3%)	0.877 (0.388−1.981)	

The ORs with analyzed by their 95% CIs were estimated by logistic regression models.
* $P < 0.05$ as statistically significant.

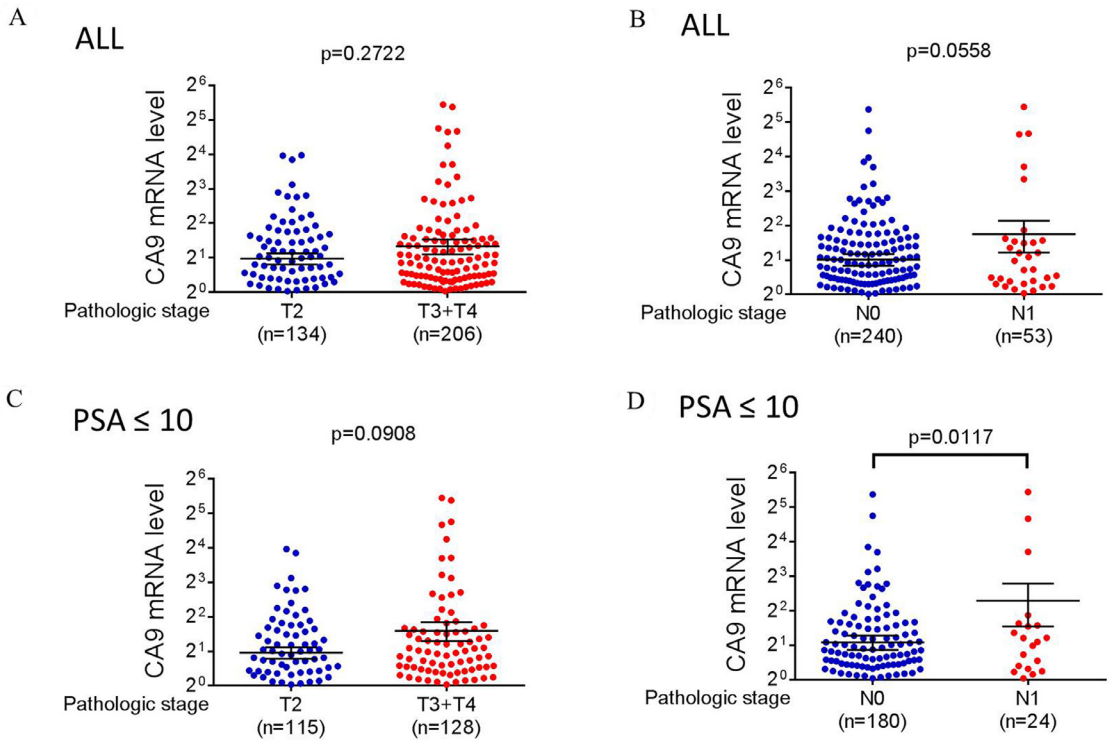

Fig. 1. CA9 mRNA level of patients with prostate cancer from the TCGA database. (A) CA9 levels were compared according to the pathological T stage in the whole population. (B) CA9 levels were compared according to the lymph node status in the whole population. (C) CA9 levels were compared according to the pathological T stage in the iPSA ≤ 10 group. (D) CA9 levels were compared according to the lymph node status in the iPSA ≤ 10 group.

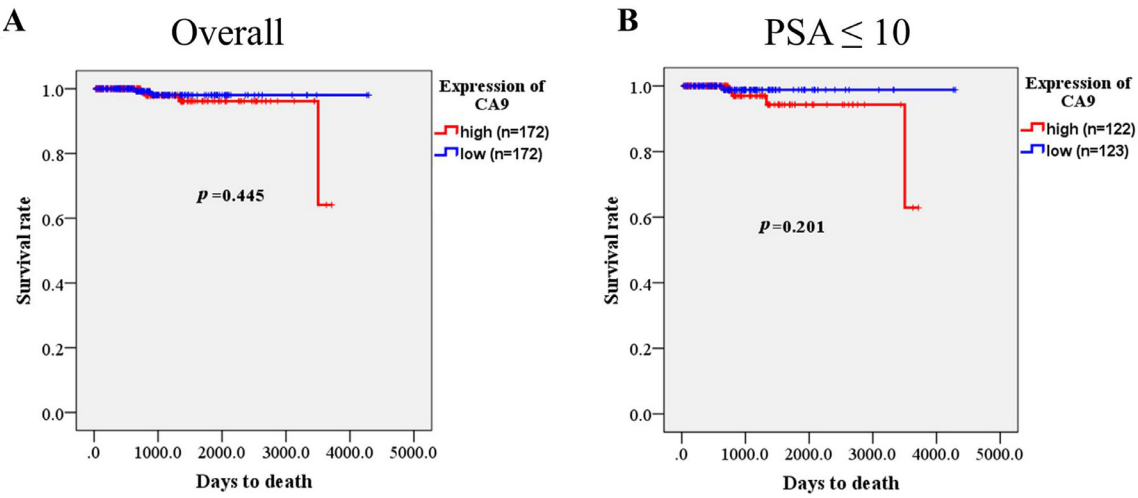

Fig. 2. CA9 mRNA level and overall survival of prostate cancer from the TCGA database. The effects of CA9 mRNA expression on the overall survival of patients with prostate cancer were evaluated with the Kaplan-Meier plot using the Log-rank (Mantel-Cox) test. The survival curve was illustrated with high (red lines) and low (blue lines) CA9 mRNA expression levels. (A) Overall patients, and (B) CaP with iPSA ≤ 10 group.

logical N1 disease than in N0 disease ($P = 0.0117$, Fig. 1D). A similar result was noted in the overall survival analysis. No difference was noted in the overall survival between high and low CA9 expression in the whole population group (Fig 2A). However, in the iPSA ≤10 group, patients with higher CA9 expression showed a trend of worse overall survival, but this was statistically nonsignificant ($P = 0.201$, Fig. 2B).

4. Discussion

PSA testing has become popular worldwide in the past decade, and only a single PSA screening intervention can boost the detection of low-risk CaP [30]. However, treatment can be deferred in up to 45% of these patients [31]. Hence, AS is a preferred option in this case. The risk of misclassifying an occult aggressive disease is the main

concern. Several genomic-based assays have been introduced for predicting recurrence after surgery, metastasis, and cancer-related death [32–36]. Several alleles were assessed to determine prognostic factors for AS [37]. CA9 was used as a marker of hypoxia in several solid tumors, including tumor of the lung, bladder, renal cells, and head and neck, [14] and moreover, hypoxia is a common feature of CaP. However, CA9 was not analyzed to determine CaP prognosis.

This is the first study to evaluate the association between CA9 SNPs and the clinicopathological features of CaP with iPSA levels ≤10 ng/ml. The results suggested that individuals with at least one G allele at CA9 rs3829078 had a 4.532 times risk of lymph node metastasis and 3.484 times risk of lymphovascular invasion in the iPSA ≤10 group. Furthermore, CA9 mRNA expression in patients with CaP in the iPSA ≤10 group significantly increased the risk of lymph node metastasis in TCGA database.

Lymphovascular invasion has been broadly reported as a prognostic factor for predicting biochemical recurrence after radical prostatectomy and radiotherapy [38–41]. The biochemical recurrence risk increased with increasing lymph node metastasis rather than the residual cancer cells of positive surgical margins [41]. Furthermore, a recent study showed that the minimal lymphatic involvement of CaP cells has a marked prognostic impact on disease progression [42]. Thus, the microenvironment of the CaP cell is a key for the further investigation of CaP prognosis.

CA9 is a transmembrane protein that regulates intra- and extracellular pH for maintaining an inhospitable microenvironment for cancer cells [9]. CA9 expression can modulate E-cadherin-mediated cell–cell adhesion through affecting beta-catenin and increased cell motility [43]. In CaP, CA9 plays a crucial role in the cancer microenvironment. In cell culture research, a molecular model demonstrated that CA9 expression in CaP-associated fibroblast mediates the epithelial-mesenchymal transition of cancer cells and subsequently increases cancer cell motility, survival, and stemness [17]. Pharmacological inhibition of CA9 activity can lead to dysregulated intracellular pH, reduced cell growth, elevated apoptotic cell death, and possibly impeded tumor metastasis [16]. In a clinical setting, CA9 expression was reported to be significantly higher in CaP cells than in benign prostatic tissue, especially in the extraprostatic disease [44]. Furthermore, a small-scale study showed that the baseline serum CA9 level in castration-resistant prostate cancer is correlated to a more aggressive disease [13].

Our results showed that CA9 expression significantly affects the spread of disease, especially in a relatively low PSA group, which implies a low tumor burden and probably early-stage CaP. Two possible explanations exist regarding no difference in CaP prognosis with high PSA. First, CA9 can affect tumor survival and metastasis. However, many other factors influence the different stages of CaP. Once the PSA level and tumor burden increase, other confounders must be considered. A more reasonable hypothesis is based on "selection bias." In our study, a significant distributional difference in terms of the rs3829078 genotype was observed between the 2 PSA groups, but all participants were Asian (mostly Taiwanese) and received robotic radical prostatectomies. However, the difference in genetic polymorphism among different ethnicities would be minimal. A reasonable explanation is that patients with CaP having at least 1 G allele at CA9 rs3829078 have a higher risk of lymph node metastasis, which cannot be treated with surgery. If these patients were diagnosed during PSA testing (>10 ng/ml), they would not be selected for surgery because of their metastatic nature.

Two extremely different reports on the CA9 expression of CaP cells have been noted. Initially, Smyth et al. reported only occasional CA9 positivity in hypoxic areas and implied an alternative pathway for maintaining the pH balance in CaP cells [14]. Later, Ambrosio et al. reported a positive correlation between CA9 expression and prognostic factors, including Gleason score, ISUP 2015 grading system, and TNM staging. Combining the aforementioned 2 results, we may infer that CA9 expression is a strong prognostic factor for CaP but is not necessary in CaP development. Therefore, a method of detecting the prevalence of CA9 expression in CaP must be devised, and the SNP of CA9 may be the key.

A trend of worse overall survival and high CA9 expression in the TCGA database was noted in the iPSA ≤10 group, although this was statistically nonsignificant. This may have resulted from a relatively small sample size and short follow-up time. Only 122 cases and 123 cases in the high- and low-CA9 expression groups were included, respectively. This sample size may be insufficient for establishing significance because the incidence of disease-related death was relatively low in these patients. Moreover, the follow-up duration was not sufficiently long. The survival curves in the first 3 years of both groups overlapped and separated thereafter. In patients with low-risk CaP, cancer-specific survival could be >10 years easily. Because of these 2 reasons, although no significant difference was observed in overall survival, we believe that CA9 expression in the iPSA ≤10 group is a prognostic factor for overall survival.

In conclusion, our results indicated that men with CaP who are potential candidates for AS (iPSA ≤ 10 group) and are carrying CA9 SNP at rs3829078 are more likely to develop lymph node metastasis and further worse prognosis.

Conflict of interest

The authors declared no conflict of interest.

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