Tissue Chromogranin A Expression during Prostate Cancer Progression: Prediction of Chemosensitivity

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Purpose: We investigated the clinical significance of chromogranin A (CgA) expression as a neuroendocrine (NE) marker during prostate cancer (PCa) progression, especially as a potential predictor of chemotherapeutic response in castration-resistant PCa (CRPC) patients based on immunohistochemical findings.

Materials and Methods: Sixteen CRPC patients who underwent combination (docetaxel/estramustine/carboplatin; DEC) chemotherapy were retrospectively studied. Immunostaining of CgA was performed using prostate biopsy samples obtained at the initial PCa diagnosis, during androgen deprivation therapy, at the time of CRPC diagnosis, and after 2 cycles of DEC therapy. The positive rate was expressed as the mean percentage of positively stained tumor cells against the total number of tumor cells. Differences in positive rates among the treatment courses were compared using a Mann-Whitney test.

Results: The mean percentage of CgA-positive PCa cells increased in a stepwise manner until CRPC development and then significantly decreased after DEC therapy. Subanalysis of CgA at CRPC diagnosis showed a more evident reduction of CgA expression after DEC therapy in patients who also had a high level of CgA as compared to those with a low CgA level (P = .003). Likewise, longer prostate-specific antigen progression-free survival was related to CRPC and high CgA (P = .028).

Conclusion: NE differentiation of PCa cells is accelerated despite androgen deprivation and reaches a peak at the time of CRPC diagnosis. Although further studies using larger samples are needed, CgA expression in CRPC may be a candidate tissue biomarker to reflect the chemotherapy sensitivity of individual PCa cells.

Keywords: prostatic neoplasms; castration-resistant; neuroendocrine cells; chromogranin A; blood.

INTRODUCTION

The normal human prostate is histologically composed of tubular and/or alveolar glands, with luminal basal and secretory cells and stromal components. Neuroendocrine (NE) cells are dendritic intraepithelial cells known to regulate both prostatic growth and differentiation,[1] thus it is not surprising that they are actively involved in the process of prostate disease.[2] Indeed, focal NE differentiation represents a common feature of prostate cancer (PCa) and occurs in 30-100% of reported cases. A synergistic functional network between epithelial prostate-specific antigen (PSA) secretory cells and the NE intra-prostatic system is the main trigger for induction and sustenance of NE differentiation.[3-5] Chromogranin A (CgA), NE-derived peptide, and levels in both serum and tissue are considered to be an excellent NE marker.[6] NE cells are thought to be resistant to androgen deprivation due to lack of an androgen receptor. Previous studies have indicated that NE-positive cells in the prostate can survive and are likely to be activated in response to androgen deprivation.[4,7,8] Thus, NE differentiation is believed to contribute to development of castration-resistant prostate cancer (CRPC).[9,10] In addition, previous studies have shown that prostatic NE differentiation is closely associated with tumor progression and poor clinical outcome.[11-13] However, few reports have addressed the issue of active changes of NE differentiation in PCa tissues obtained from the same individual. Taxane-based chemotherapy has become a standard first-line therapy for CRPC,[14,15] while platinum-based chemotherapy has a cytotoxic effect on NE cells.[16,17] Therefore, combination chemotherapy with taxane and a platinum derivative is considered to be an attractive approach for treating patients with CRPC, in whom NE-positive PCa cells are likely to be activated. Indeed, we previously reported excellent clinical outcomes with such a chemotherapy combination (docetaxel /estramustine /carboplatin; DEC therapy) in patients with CRPC.[18] Based on the hypothesis that cancer cells with NE differentiation are actively involved in the process of castration resistance in PCa and sensitive to platinum-based chemotherapy, we considered that analysis of NE differentiation of PCa cells would contribute to prediction of therapeutic response and survival benefit in CRPC patients treated with DEC therapy. The purpose of this study was to evaluate tissue alterations of CgA in the same individuals during their treatment course; namely at initial diagnosis, during androgen deprivation therapy (ADT), at the time of diagnosis of CRPC, and after 2 cycles of DEC

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chemotherapy. In addition, we assessed whether the expression of CgA in affected tissues is a potential predictor of chemotherapeutic response in CRPC patients.

MATERIALS AND METHODS

Patient Selection

For this retrospective analysis, we examined data obtained from 53 CRPC patients who underwent DEC therapy between October 1999 and April 2005 at our institution. Patients were evaluated for response using samples from systematic sextant biopsies of the prostate at the time of CRPC diagnosis and after 2 cycles of DEC therapy. Access for the biopsy was transrectal and the bioptic scheme included a minimum of 8 peripheral cores. We excluded 35 patients who did not undergo a prostatic biopsy at the initial PCa diagnosis performed at our institution and 2 who did not undergo that after 2 cycles of DEC therapy, yielding a 16-patient cohort. **Figure 1** diagrams the times of biopsy, the number of patients in this cohort for analysis and the number of ineligible patients. Among these 16 patients, 7 underwent several prostate biopsies at the initial PCa diagnosis, during ADT, at CRPC diagnosis, and after 2 cycles of DEC chemotherapy, while the remaining 9 underwent prostate biopsies at the same time points, except for during ADT. Informed written consent was obtained from all patients after receiving institutional review board approval. All study protocols were approved by the ethics committee of Shimane University Faculty of Medicine in accordance with the 1975 Declaration of Helsinki (20140919-2).

Table 1. Demographic and clinical characteristics of 16 patients.

Variables	n = 16
Age (years), median (range)	72 (52-86)
Performance statues, no (%)	
0-1	12 (75.0)
2-3	4 (25.0)
PSA value at initial PCa diagnosis, ng/mL median (range)	142.4 (0.8-6113.9)
Gleason score, no (%)	
7	3 (18.7)
8	2 (12.5)
9	11 (68.8)
Duration of initial hormone therapy, months median (range)	18.6 (4.1-50.7)
Measurable extraosseous disease, no (%)	
Negative	7 (43.8)
Positive	9 (56.2)
Lymph nodes	7
Liver	3
Lung	2
Osseous disease, no (%)	
Negative	2 (12.5)
Positive	14 (87.5)
Hormone therapy, no (%)	
Maximum androgen blockade	16 (100)
LH-RH analogue	11 (68.8)
Surgical castration	5 (31.2)

Abbreviations: PSA, prostate specific antigen; PCa, prostate cancer; LH-RH, luteinizing-hormone releasing hormone.

Treatment Regimen of DEC Therapy

Eligibility criteria for DEC chemotherapy were as follows: 1) Eastern Cooperative Oncology Group performance status (PS) score of 0-3; 2) baseline leukocyte count greater than 3000/μL; 3) hemoglobin 8.0 g/dL or greater; 4) platelet count exceeding 100,000/μL; 5) adequate renal function defined as serum creatinine 1.5 times or less than the upper limit of normal (ULN); 6) adequate liver function defined as bilirubin less than ULN and aspartate transaminase less than 1.5 times ULN, 7) adequate cardiac function; 8) life expectancy of more than 3 months; and 9) more than 8 weeks elapsed since any major surgery, radiotherapy, or prior chemotherapy. The DEC therapy was comprised of weekly intravenous administrations of docetaxel at 30 mg/m^2, daily oral estramustine at 10 mg/m2, and intravenous carboplatin every 28 days to reach an area under the curve value of 6 on day 1 of every 4-week cycle.[18] CRPC was defined as three increases in the PSA level at least 1 month apart, or evidence of a new clinical disease despite discontinuation of antiandrogen (androgen withdrawal) medication.[19] During DEC therapy, ongoing ADT was also applied. Pretreatment evaluation procedures included medical history, physical examination, complete blood count, and chemistry profile, serum PSA, alkaline phosphatase, and lactate dehydrogenase levels, 24-hour creatinine clearance, and 12-lead electrocardiogram, chest X-ray, bone scintigraphy, computerized tomography (CT) scan, and magnetic resonance imaging findings. Treatment was continued until disease progression, an unacceptable adverse event, or patient refusal occurred.

Clinical Evaluation of DEC Therapy

Response rate was determined according to standard phase II response criteria[19] on the basis of imaging findings, including chest X-ray, CT scan, and bone scintigraphy, at least every 8 weeks for 4 cycles. Complete response (CR) was defined as complete disappearance of all disease and partial response (PR) as ≥ 50% reduction in the sum of the values for the perpendicular diameters of all lesions. Stable disease (SD) was defined as < 50% reduction or ≤ 25% increase in the sum of the values for the perpendicular diameters of all lesions. Since changes in intensity or sizes of osseous lesions using bone scanning are difficult to interpret, the appearance of 1 or more new osseous lesions was required on bone scans to identify progressive disease. PSA levels were measured every 4 weeks. PSA progression was defined as 3 consecutive increases in that level of at least 50% over the nadir value at a minimum of 4 ng/mL. Time to PSA progression was calculated from the first day of CRPC treatment to the final day of the study or evidence of progressive disease. Cause-specific survival was determined from the initiation of DEC therapy to the day of death or last follow-up examination.

Immunohistochemistry

Biopsy samples were fixed in 10% buffered formalin (pH 7.0) for 12 hours and embedded in paraffin wax, then 5 consecutive 5 μm sections were cut from each block and used for hematoxylin and eosin staining for

Table 2. Correlation of neuroendocrine differentiation with Gleason score and serum PSA value.

Variables	Mean CgA Expression ± SD (range)	*P* Value
Initial PCa diagnosis		
Gleason score		
≦ 8 (n = 5)	8.24 ± 6.97 (0-17.05)	.610
> 8 (n = 11)	6.73 ± 5.22 (1.55-17.65)	
PSA value, ng/mL		
≦ 142.4 (n = 8)	8.26 ± 5.74 (0-17.65)	.345
> 142.4 (n = 8)	6.14 ± 5.05 (1.55-17.05)	
CRPC diagnosis		
Gleason score		
≦ 8 (n = 5)	15.17 ± 5.88 (10.50-25.05)	.428
> 8 (n = 11)	19.69 ± 10.20 (10.10-41.45)	
PSA value, ng/mL		
≦ 91.7 (n = 8)	20.54 ± 11.52 (10.50-41.45)	.462
> 91.7 (n = 8)	16.02 ± 5.85 (10.10-25.05)	

Abbreviations: PCa, prostate cancer; PSA, prostate specific antigen; CRPC, castration resistant PCa; CgA, chromogranin A.

Table 3. Clinical characteristics of high and low CgA groups at time of CRPC diagnosis.

Variables	High CgA Group (n = 8)	Low CgA G roup (n = 8)	*P* Value
Age (years), median	72.0	72.5	.495
PS, median (range)	0.5 (0-3)	1 (0-3)	.350
Gleason sum, median	9	9	.590
Duration until CRPC (days), median	545	557	.833
Laboratory data, median (range)			
Hemoglobin (g/dL)	12.9 (10.5-14.8)	11.1 (9.1-14.3)	.120
ALP (IU/L)	312.5 (180-466)	350.5 (286-536)	.216
LDH (IU/L)	203.5 (132-447)	222.5 (161-732)	.418
Ca++ (mg/dL)	9.3 (8.9-9.7)	9.3 (8.2-9.6)	.512
PSA value (ng/mL), median			
Initial PCa diagnosis	358.7	142.4	.833
CRPC diagnosis	35.9	129.6	.074
After 2 cycles of chemotherapy	1.7	7.7	.156
PSA decrease after 2 cycles of chemotherapy no (%)			
90 or greater	50.0 (4/8)	50.0 (4/8)	-----
Clinical outcome of measurable disease PR + CR no (%)			
Lymph nodes	80.0 (4/5)	100 (2/2)	.495
Liver	100 (1/1)	100 (2/2)	-----
Lung	100 (2/2)	-----	-----
Bone	14.3 (1/7)	12.5 (1/8)	.919
Chemotherapy (more than 10 cycles) no (%)	62.5 (5/8)	37.5 (3/8)	.317
CgA expression at initial PC diagnosis (%)	8.7	3.3	.027

Abbreviations: PS, performance status; CRPC, castration resistant prostate cancer; ALP, alkaline phosphatase; LDH, lactate dehydrogenase, Ca++, calcium; PSA, prostate specific antigen; PR, partial response; CR, complete response; CgA, chromogranin A.

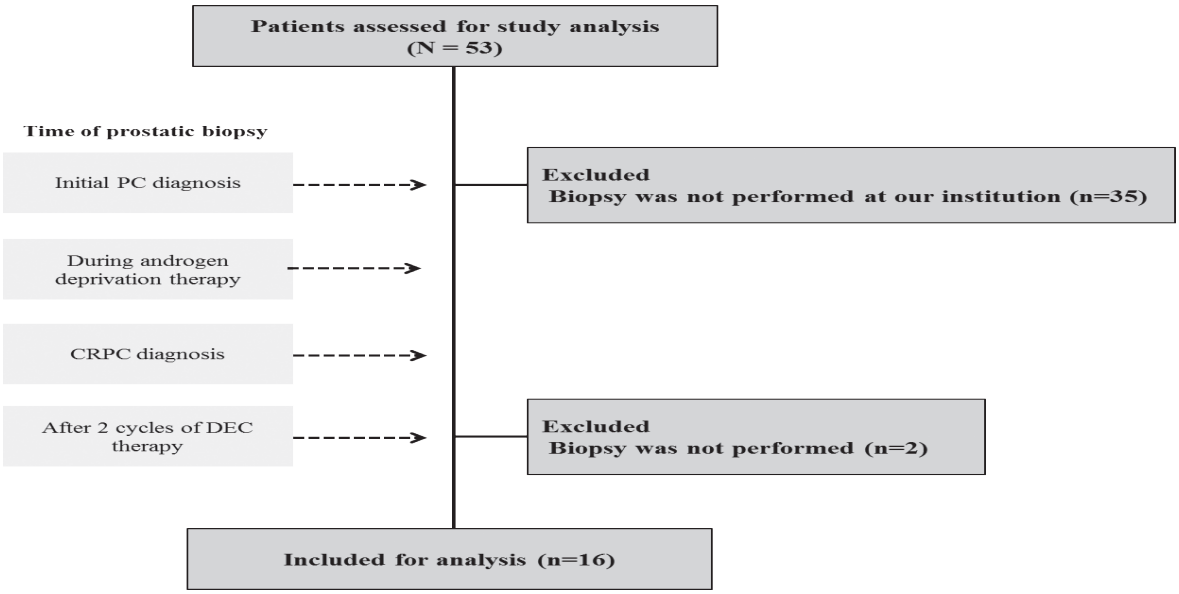

Figure 1. Flow chart detailing the times of biopsy and the available patient cohort in this study.

histological evaluation or immunostaining. CgA immunohistochemistry was performed using a rabbit polyclonal antibody raised against CgA (DAKO, Kyoto, Japan). Each slide was de-paraffinized in xylene and rehydrated through graded concentrations of ethanol in water. Endogenous peroxidase activity was blocked by incubation for 10 minutes with 3% hydrogen peroxide. Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped.

Evaluation of Immunostaining

All slides were independently reviewed by an experienced pathologist (Y.H), who was blind to all clinical data. At least 200 tumor cells found in 10 randomly selected high-power fields of each slide were examined. The positive rate was expressed as the mean percentage of positively stained tumor cells against the total number of tumor cells, as noted in our previous study.[20]

Statistical Analysis

Statistical analysis was performed using a Mann-Whitney *U* test, a χ^2 test, or log-rank test. Correlation analysis was performed using Pearson's coefficient correlation. Survival curves were conducted using the Kaplan-Meier method, with the differences between curves analyzed using a log rank test. A two-tailed *P* value of less than .05 was considered to be statistically significant.

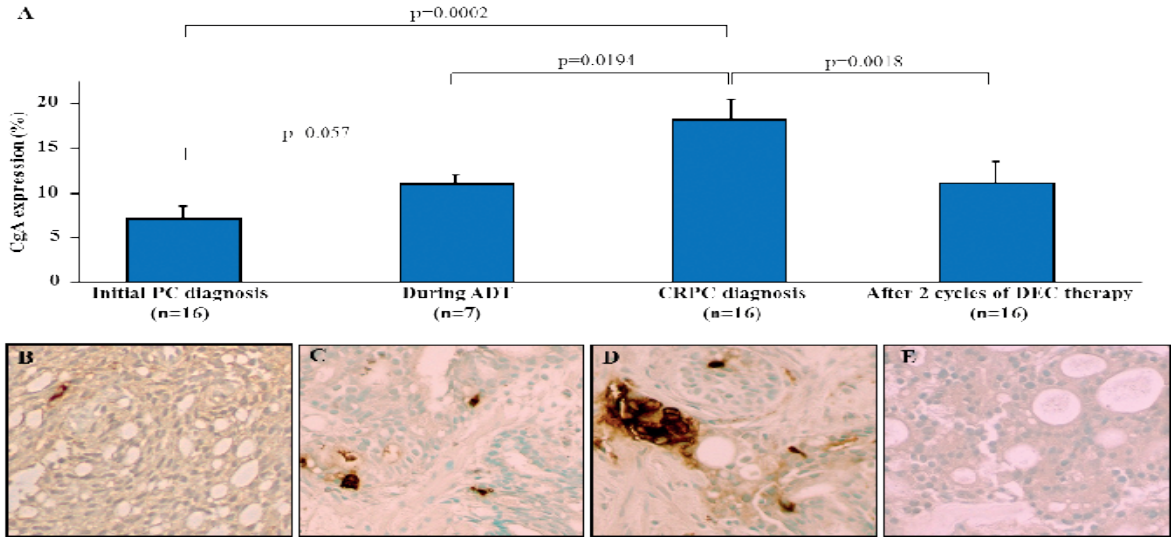

Figure 2. Cg A expression in PCa cells at the time of initial PCa diagnosis, during ADT, at the time of CRPC diagnosis, and after 2 cycles of DEC therapy. **(A)** CgA expression increased in a stepwise manner until CRPC diagnosis, then significantly decreased after 2 cycles of DEC chemotherapy. Representative immunostaining for CgA from the same patient **(B)** at the time of initial PCa diagnosis, **(C)** during ADT, **(D)** at the time of CRPC diagnosis, and **(E)** after 2 cycles of DEC chemotherapy. Magnification, × 200.
Abbreviations: CgA, chromogranin A; PCa, prostate cancer; CRPC, in castration-resistant prostate cancer; DEC, docetaxel/estramustine/carboplatin; ADT, androgen deprivation therapy.

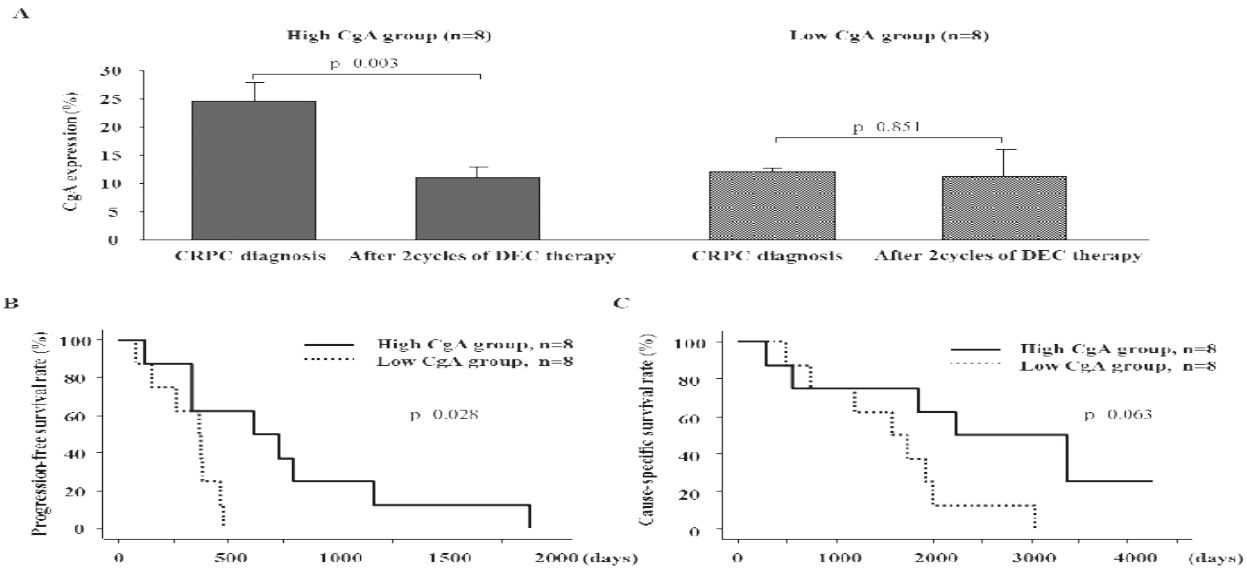

Figure 3. Subanalysis of CgA expression at time of CRPC diagnosis.
(A) A significant reduction in CgA expression after 2 cycles of DEC chemotherapy was seen in the high CgA expression group, while that was not evident in the low CgA expression group. **(B)** Patients in the high CgA group at the time of diagnosis of CRPC showed a significant longer PSA progression-free survival period as compared with those in the low CgA group at the time of diagnosis of CRPC. **(C)** Patients with high CgA at CRPC diagnosis showed a longer cause-specific survival period than the Low CgA group, though the difference did not reach statistical significance.
Abbreviations: CgA, chromogranin A; PCa, prostate cancer; CRPC, in castration-resistant prostate cancer; DEC, docetaxel/estramustine/carboplatin.

RESULTS

Patients' Profiles

Clinical characteristics of the 16 patients are shown in **Table 1**. Their ages ranged from 52 to 86 years old, with a median of 72 years. Twelve patients had a PS score of 0 or 1 and the remaining 4 had a score of 2 or 3. PSA level at the time of initial diagnosis ranged from 0.8 to 6113.9 ng/mL, with a median of 142.4 ng/mL. Of the 16 cases, 3 (18.7%) were Gleason score 7, 2 (12.5%) were Gleason score 8, and 11 (68.8%) were Gleason score 9 at the initial PCa diagnosis. During treatment, one case showed Gleason score upgrade from 7 to 8. Bidimensionally measurable extraosseous disease was preset in 9 (56.2%) patients (7 had lymph nodes, 3 had multiple liver metastases, and 2 had multiple lung metastases) and 14 (87.5%) demonstrated bone metastasis at the time of CRPC diagnosis. For the initial treatment, all patients underwent ADT by medical or surgical castration with anti-androgen. Following the diagnosis of CRPC, they were treated with DEC therapy, ranging from 3 to 35 cycles, with a median of 10 cycles. CgA Expression and Clinicopathological Findings Of the 16 patients, 1 (6.3%) had CgA negative tumor cells and 12 (75%) immunoreactive neoplastic cells under 10% at the initial PCa diagnosis. There were no pure NE carcinomas of the prostate such as small cell carcinoma or carcinoid. The mean percentage of PCa cells with positive CgA expression at the initial PCa diagnosis, during ADT, at CRPC diagnosis, and after 2 cycles of DEC chemotherapy were 7.2%, 11.0%, 18.3% and 11.1%, respectively. Thus, CgA expression increased in a stepwise manner until diagnosis of CRPC, while it was significantly decreased after 2 cycles of DEC chemotherapy (**Figure 2A**). Representative alterations of CgA immunostaining in the same patient are shown in **Figures 2B-E**. **Table 2** shows the correlation of NE differentiation with Gleason score and serum PSA value. CgA expression at the initial PCa diagnosis or CRPC diagnosis was not associated with Gleason score in each stage. In addition, after dividing the 16 cases into 2 groups according to median serum PSA value at the initial PCa (142.4 ng/mL) or CRPC diagnosis (91.7 ng/mL), there was no significant correlation between serum PSA level and CgA expression in each stage. Prognostic Relevance of CgA Expression for Development of CRPC in 16 Cases Treated with DEC Therapy Next, we classified the 16 patients who underwent DEC therapy into 2 groups according to the median percentage of CgA positive PCa cells at CRPC; namely the high and low CgA groups. The clinical characteristics of both groups are summarized in **Table 3**. There were no significant differences for age, PS score, Gleason score, duration until CRPC, hemoglobin, alkaline phosphatase, lactate dehydrogenase, or serum calcium between the groups. Although there was a trend that PSA value at the time of CRPC diagnosis in the Low CgA group was higher than that of high group, the number of cases with a PSA reduction rate of more than 90% was the same in 2 groups. Of 5 assessable patients with lymphadenopathy, 4 (80%) attained PR or CR, and of 3 patients with measureable liver or lung metastasis, 3 attained PR or CR in the high CgA group. The patients having lymphadenopathy or liver metastasis in the low CgA group could also attain PR or CR. Of 7 patients with positive bone metastasis in the high CgA group, bone scan revealed improvement in only 1 (14.3%). Similarly, the bone response rate in the low CgA was only 12.5%. Thus, the response rate for measurable lesions in each group was approximately equivalent. DEC therapy of more than 10 cycles was

more prevalent in the high than the Low CgA group (62.5% vs. 37.5%), though the difference was not statistically significant. Interestingly, CgA expression at the initial PCa diagnosis was significantly higher in the high CgA group than in the low CgA group ($P = .027$). As shown in **Figure 3A**, a significant reduction in CgA-positive PCa cells was found after 2 cycles of DEC chemotherapy in the high CgA group ($P = .003$), whereas no such reduction was found in the low CgA group. There was no significant correlation between the change of PSA value and change of CgA expression score (data not shown). The median periods of PSA progression-free and cause-specific survival were 378 and 1885 days, respectively. PCa patients in the high CgA group at the time of CRPC diagnosis showed a significantly longer PSA progression-free survival period as compared with those in the low CgA group at CRPC diagnosis ($P = .028$; **Figure 3B**). Likewise, the high CgA group at diagnosis showed a longer cause-specific survival period than the low CgA group, though the difference did not reach statistical significance ($P = .063$; **Figure 3C**).

DISCUSSION

Previous studies found that PCa cells with NE differentiation are likely to be increased and functionally accelerated after acquiring castration resistance.[4,7,8] However, few reports have addressed the issue of active changes of NE markers in PCa tissue obtained from the same individuals. In the current study, we focused on tissue alterations of CgA during the course of treatment and report the clinical potential of NE differentiation in PCa. As shown in **Figure 2A**, PCa cells with NE differentiation were activated despite androgen deprivation in a stepwise manner until acquisition of castration resistance. In addition, we found that NE differentiation of PCa cells is not correlated with PSA value, as previously reported in the literature.[8,12] In parallel with the acquisition of castration resistance, PCa cells with NE differentiation are increased due to their survival capability despite androgen deprivation. Thus, it is possible that PCa cells with NE differentiation have an association with acquisition of castration resistance. Although taxane-based chemotherapy is now considered to be a standard first-line chemotherapy for CRPC, the median progression-free survival period appears to be less than 7 months.[21] Since combination chemotherapeutic strategies such as DEC therapy have been shown to have more prognostic relevance than conventional taxane-based chemotherapy, an NE-targeted chemotherapeutic strategy may be an attractive alternative to taxane-based chemotherapy for CRPC patients. On the basis of findings that active involvement of NE differentiation is related to the process of castration resistance in PCa, NE cells are considered to be chemosensitive to carboplatin, as shown in studies of small cell lung cancer.[22] Also, the combination of taxane-based chemotherapy and carboplatin confers an excellent prognostic relevance for patients with CRPC.[18,23] Thus, we propose that therapeutic response to DEC therapy as well as survival benefit in CRPC patients may be predictable by determining PCa cells with NE differentiation just prior to DEC therapy. The present results showed that the reduction in NE-positive PCa cells after 2 cycles of DEC therapy was more significant in the high CgA expression group than the low CgA expression group. Likewise, better PSA progression-free probability was noted in CRPC patients with a high level of CgA expression. Together, these findings suggest that CgA expression in tissue at the time of CRPC diagnosis may be useful as a biomarker for prediction of chemosensitivity. Although no significant survival benefit was demonstrated in our study, cause-specific survival was longer in the High CgA group at CRPC diagnosis. As shown in **Table 3**, 62.5% of the cases in the high CgA group underwent DEC therapy for more than 10 cycles, as compared to only 37.5% in the low CgA group. Therefore, we believe that CRPC patients with a high level of CgA expression can have DEC therapy for a longer period because of delayed disease progression. PCa is now the second leading cause of death in the United States of America,[24] and this notorious propensity is applicable to Japan. In general, PCa shares the characteristics of slow growth with a longer lifespan as compared to other types of cancer. However, despite radical treatment, PCa patients harboring a biologically aggressive phenotype, such as those with positive NE differentiation, may unfortunately progress into early disease recurrence and ultimately death. In the present study, we found that PCa cells with a higher level of CgA expression at the time of CRPC diagnosis also had higher CgA expression at the initial PCa diagnosis (**Table 3**). A finding of persistently elevated CgA expression despite ADT in PCa tissue might provide rationale for the neoadjuvant modality of platinum-based chemotherapy prior to radical treatment for PCa patients, especially those with higher CgA expression at the initial diagnosis, in order to prevent an unfavorable clinical outcome. There are several limitations in this study. First, the small number of cases analyzed retrospectively. Our results demonstrated that the duration of ADT is not associated with CgA expression at CRPC diagnosis. In contrast, Abrahamsson and colleagues[25] reported that induction of NE differentiation was strongly related to the duration of ADT. Previous studies have reported an association of NE differentiation and a high Gleason score[11,12,26] although we and other investigators failed to detect this relationship.[13,27,28] In addition, the prognostic significance of NE differentiation in PCa is not well elucidated and controversial because most articles on the issue include relatively small number size. Second, CgA expression was performed by immunohistochemical staining using prostate biopsy samples. Transrectal ultrasound guided prostate biopsy is associated with a certain degree of under- and overstaging of PCa, and it may fail to assess the definite tumor burden in the patients with metastasis. The measurement of serum CgA may avoid tumor heterogeneity and tissue sample biases because it corresponds to the entire primary tumor cell population and its associated metastases.[27] Furthermore, in addition to NE differentiation, there are other mechanisms contributing to development of CRPC and prognosis such as multiple pathways related to androgen receptor or apoptosis related to the Bcl-2 family[20,29,30] which were not evaluated in this study. Taking into consideration these limitations, the validity of our results should be cautiously considered. Thus, further research using a larger number of cases is required to verify our findings.

CONCLUSION

NE differentiation of PCa cells is accelerated despite androgen deprivation and reaches a peak at the time of

CRPC diagnosis, suggesting active involvement of NE differentiation in acquisition of castration resistance in affected patients. Early prediction of chemosensitivity using tissue CgA expression may provide a beneficial effect for CRPC patients undergoing combination taxane chemotherapy with a platinum derivative, such as DEC therapy. However, further research using a larger number of cases is needed to clarify our findings.

CONFLICTS OF INTEREST

None declared.

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