An immunohistochemical study of chromogranin A and human epidermal growth factor-2 expression using initial prostate biopsy specimens from patients with bone metastatic prostate cancer

Yoshiaki Yamada, Kogenta Nakamura, Shigeyuki Aoki, Tomohiro Taki, Katsuya Naruse, Hiroyuki Matsubara, Motoi Tobiume, Kenji Zennami, Remi Katsuda and Nobuaki Honda

Department of Urology, Aichi Medical University School of Medicine, Nagakute-cho, Aichi, Japan

Accepted for publication 10 July 2006

OBJECTIVES

To investigate, using prostate needle-biopsy specimens at diagnosis from patients with bone metastatic prostate cancer, whether the relationship between neuroendocrine (NE) cell differentiation and human epidermal growth factor-2 (HER-2) expression is a prognostic factor for outcome.

PATIENTS AND METHODS

The study included 50 patients diagnosed as having bone metastatic prostate cancer between January 1998 and December 2001. We tested for NE cell differentiation by using immunohistochemical (IHC) staining for chromogranin A (CgA), and for HER-2, using a commercial test for IHC staining.

CONCLUSIONS

NE cell differentiation of the primary tumour in patients with bone metastatic prostate cancer does not reflect the prognosis, whereas HER-2 overexpression is a prognostic factor for an unfavourable outcome. These results suggest that NE cell differentiation is not induced by HER-2.

KEYWORDS

bone metastatic, prostate cancer, chromogranin A, neuroendocrine differentiation, HER-2, immunohistochemistry, prognosis

INTRODUCTION

Neuroendocrine (NE) cell differentiation has been identified as one factor involved in the acquisition of resistance to endocrine therapy in prostate cancer [1]. NE tumour cells do not express androgen receptors, and do not proliferate in response to androgen [2]. Thus they are thought to be resistant to endocrine therapy with androgen ablation. In addition, a mechanism has been postulated whereby the NE substance secreted by NE tumour cells stimulates the proliferation of the surrounding tumour cells via the paracrine system [2]. It was shown that chromogranin A (CgA) is most universally expressed in NE cell differentiation [3,4]. Many published reports indicated that in both immunohistochemical (IHC) investigations [3,5,6] and measurements of blood serum concentration [7–9], CgA is a significant prognostic factor for outcome after endocrine therapy.

Human epidermal growth factor receptor 2 (HER-2/neu, or HER-2) is a proto-oncogene on chromosome 17q21 that encodes a transmembrane tyrosine growth factor. Basic research has shown that HER-2 overexpression induces malignant transformation in cells and increases aggressiveness. Since Slamon et al. [10] first reported the correlation between amplification of the HER-2 (c-erbB-2) and poor-prognosis breast cancer in 1987, HER-2 has been considered among the unfavourable prognostic factors for breast cancer, and breast cancer with overexpression of HER-2 is reportedly resistant to hormone therapy [11,12].

Some investigators have attributed growth factor-like activity to some NE products [3,13], and Iwamura et al. [14] reported that NE cells in normal prostate tissue might be regulated by the HER protein family, probably in a ligand-specific fashion, using IHC staining methods.

In the present study we investigated the expression of NE cell differentiation using IHC staining for CgA, and the HER-2 oncprotein by using a commercial test for IHC [15], on diagnostic prostate needle-biopsy specimens from patients with bone metastatic prostate cancer. We sought a correlation between NE cell differentiation and HER-2 expression, and investigated whether this could be a prognostic factor for outcome.

PATIENTS AND METHODS

The study included 50 patients examined at the authors’ institution between January 1998 and December 2001, and who had been diagnosed with bone-metastatic stage D2 prostate cancer. The median (range) period of
observation was 48.7 (6.9–79.4) months. The treatment method was maximal androgen blockade, with patients receiving antiandrogen agents, with LHRH agonists in 47 and antiandrogen agents in addition to bilateral orchidectomy in three. In all patients PSA levels decreased to <4.0 ng/mL after maximal androgen blockade. None of the patients had received any anticancer agents after recurrence. This research was approved by the Institutional Review Boards (No. 275) of Aichi Medical University, and full informed consent was obtained from all patients before staining for CgA and HER-2; Table 1 shows the patients' characteristics.

PSA levels were measured using the Hybritech system (San Diego, CA, USA). For prostate biopsy, an Aloka (Tokyo, Japan) SSB-650 CL TRUS system was used to guide systematic sextant biopsy. The clinical stage and extent of disease (EOD) grade [16] were determined by CT, MRI and bone scans.

Differences between groups were tested for significance using the Mann–Whitney U-test and the chi-squared test, with P < 0.05 considered to indicate significance. The survival and no-recurrence periods were calculated using the Kaplan–Meier method, with significant differences assessed using the log-rank test. The day on which the clinical stage was determined was regarded as the first day of observation (day 0). Recurrence was regarded as biological failure and determined by three consecutive increases in PSA level, with the first counted as the date of recurrence. The final date of observation was 31 December 2004. The Gleason classification [17] was used for histopathological grade, and the TNM classification [18] to evaluate the primary tumour and lymphatic metastasis.

We used the labelled streptavidin-biotin method of immunostaining for identifying CgA. Paraffin sections 5 μm thick, from biopsy tissue samples taken at the time of original diagnosis, were washed with PBS (pH = 7.4) after paraffin removal and treatment with 0.3% H2O2 in methanol to block endogenous peroxidase activity. Nonspecific reactions were blocked by 5% normal goat serum. A 1 : 400 dilution of rabbit anti-human CgA (DAKO Cytomation, Glostrup, Denmark) was used as the primary antibody, which was reacted for 30 min at 37 °C. After washing with PBS they were reacted for 30 min at 37 °C with horseradish peroxidase-labelled streptavidin, and washed with PBS.

### Table 1 The patients' characteristics stratified by CgA and HER-2 IHC staining results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CgA</th>
<th>HER-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Number of patients (%)</td>
<td>11 (22)</td>
<td>39 (78)</td>
</tr>
<tr>
<td>Age, years:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>73.9 (6.1)</td>
<td>73.3 (7.8)</td>
</tr>
<tr>
<td>median (range)</td>
<td>76 (63–82)</td>
<td>72 (61–91)</td>
</tr>
<tr>
<td>Serum PSA level, ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (sd)</td>
<td>1016.0 (1217.5)</td>
<td>1037.8 (2035.1)</td>
</tr>
<tr>
<td>median (range)</td>
<td>440 (50.0–3780)</td>
<td>270 (34.0–10060)</td>
</tr>
<tr>
<td>Number of patients:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>EOD grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1c</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>T3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>T4</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Tx</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>N1</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>N2</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

No differences were statistically significant.
Sections were subsequently developed in diaminobenzidine and counterstained with haematoxylin for nuclear staining. The specimens were examined by two experienced pathologists and considered positive if the positively stained cells comprised ≥10% of the tumour area [5] (Fig. 1). Paraffin sections 5 µm thick were stained for expression of the HER-2 protein using a standardized Hercep test (DAKO Cytomation, Carpinteria, CA, USA) The primary antibody was an affinity-purified rabbit polyclonal antiserum raised against an intracellular epitope of the human HER-2 molecule (specification sheet K5205, Dako). The staining procedure includes an antigen-retrieval step consisting of a 40-min incubation in pH 6.0 citrate buffer (Target Retrieval, Dako) in a water bath at 99 °C. Sections were stained on an automated staining apparatus for IHC (Autostainer, Dako) according to the manufacturer’s guidelines. Slides were counterstained with haematoxylin. Each immunohistochemical staining experiment included a set of control slides. For the negative control section the primary antibody was replaced with an irrelevant, isotype-matched antibody, to control for nonspecific binding of the secondary antibody reagent. The positive control slide consisted of sections of cell blocks of the three breast cancer cell lines SKBR3, MDA-MB-175 and MDA-MB-231, which express 2.4 million, 92 000 and 22 000 HER-2 receptor molecules, by Scatchard analysis, respectively. These receptor numbers were evaluated by two pathologists and classified into four levels according to the classification of Carter et al. [19]. A score of ≥+ or above was counted as positive (Fig. 2).

RESULTS

There were CgA-negative cells in 29 patients, eight were 1–5% positive, two were 5–9% positive, and 11 had staining of >10% of their tumour and were regarded as CgA-positive. A comparison of clinical data for the CgA-positive and -negative groups at the time of diagnosis showed no significant differences in serum PSA level, EOD grade, Gleason score, T stage, or N stage (Table 1).

The 5-year cause-specific survival rate was 34.1% for the CgA-positive group and 55.2% for the CgA-negative group, but with \( P = 0.376 \) this was not a significant difference (Fig. 3A). The 3-year no-recurrence rate was 9.1% for the CgA-positive group and 35.9% for the CgA-negative group, and this difference was significant (\( P = 0.025 \); Fig. 3B). There was a recurrence in all 11 patients in the CgA-positive group and 30 of 39 (77%) in the CgA-negative group. The 3-year cause-specific survival rates were 38.4% and 42.3%, respectively, but with \( P = 0.813 \) this difference was not significant (Fig. 3C).

HER-2 staining scores were 0 in 28 patients, + in four, ++ in 11 and +++ in six, with one indeterminate. Counting scores of ≥+ as HER-2 overexpression, the HER-2 positive group comprised 21 patients (42%). Excluding the one indeterminate patient, comparing the HER-2-positive and -negative groups for the remaining 49 on the date of clinical diagnosis showed no significant differences in serum PSA level, EOD grade, Gleason score, T stage or N stage (Table 1).

The 5-year cause-specific survival rate for the HER-2 positive group was 21.2%, whereas that for the HER-2-negative group was 63.0%, a significant difference (\( P = 0.008 \); Fig. 4A). The 3-year no-recurrence rate was 23.8% in the HER-2-positive and 37.5% in the HER-2-negative group, a significant difference (\( P = 0.049 \); Fig. 4B). There was a recurrence in 19 of 21 patients (91%) in the HER-2-positive, and 21 of 28 (75%) in the HER-2-negative group. The 3-year cause-specific survival rates were 23.4% and 48.0%, respectively. The survival rate after recurrence was significantly higher for the HER-2-negative group, with \( P = 0.025 \) (Fig. 4C).

When ++ and +++ scores were considered positive, as in the case of breast cancer, the 5-year cause-specific survival rate was 14.7% in the HER-2-positive and 60.6% in the HER-2-negative group (\( P = 0.007 \)). The 3-year no-recurrence rate was 11.8% in the HER-2-positive and 37.5% in the HER-2-negative
group ($P = 0.006$). The survival rate after recurrence was also greater in the HER-2-negative group ($P = 0.024$).

We assessed the 49 patients in whom both factors could be determined; six were positive for both (12%), four were positive for CgA and negative for HER-2 (8%), 15 were negative for CgA and positive for HER-2 (31), and 24 were negative for both (49%). There was no statistically significant correlation ($P = 0.220$).

**DISCUSSION**

NE cells are found within prostate tissue; they extend dendrites between their neighbouring epithelial cells, and their cytoplasm includes granules of accumulated peptide hormones and pro-hormones. The function of NE cells within the prostate is unknown, but conjecturing from the functions of NE cells in the respiratory and digestive systems and in the pancreas leads to the consideration that they are indispensable to the growth and differentiation of the prostate, as well as to the homeostatic regulation of the endocrine process [1]. Several immunohistological immunoassay investigations of prostate cancer tissue have been reported [4,20]. For prostate tissue in particular, CgA was reported as being the most universally expressed during NE cell differentiation [21]. Studies have been reported for IHC staining for CgA in prostate cancer tissue, and research on NE cell differentiation according to serum CgA measurements [22].

The study by Bostwick et al. [23] used the number of positive cells as the criterion for a positive determination on IHC staining. However, as there are few CgA-positive cells in normal prostate epithelium, for the present study we considered staining positive if positive cells accounted for >10% of the surface area of the tumour [5].

McWilliam et al. [5] and Krijnen et al. [6] reported that the existence of NE cells is an independent prognostic factor for outcome. However, their studies included patients at different stages of disease, and as they are unlike the present study of one stage they cannot be evaluated in the same way. Aprikian et al. [24] and Bostwick et al. [23] studied patients with advanced prostate cancer and lymph node metastasis, and reported no significant difference in survival rate with NE cell differentiation. In their in vivo research...
using PC-310 human prostate cancer xenograft cells, Jongsm et al. [25] also reported that androgen-dependent tumours shrank by apoptosis within a few days after castration, with half the surviving cancer cells being positive for CgA. They concluded that a proportion of tumour cells that had initially been androgen-dependent had differentiated into NE cells with no accompanying tumour proliferation after androgen ablation.

Bonkhoff [2] also reported that NE cells had undergone their final differentiation and had no potential for proliferation. In addition, an in vitro study using prostate cancer cells reported that CgA inhibits the invasion and growth of prostate cancer cells [26]. These reports show that in patients with bone metastatic prostate cancer and with a short time to recurrence, as the cancer cells are highly biologically malignant, it is possible that CgA secretion increases in an attempt to suppress tumour proliferation through NE cell differentiation and inhibit the growth of tumour cells. This might be why there was no significant difference in survival rates, despite the significantly shorter period to recurrence.

The present results showed HER-2 overexpression in 42% of patients. In the study by Ross et al. [27] of HER-2 in prostate cancer, using IHC, the rate of overexpression was 29%, which they reported to correlate with clinical grade. However, the present data showed no correlation with clinical grade. Signoretti et al. [28] further reported that HER-2 overexpression increased as the disease progressed into androgen independence or hormone resistance, in 25% (17/67) of patients who had surgery alone, in 59% (20/34) of those who had antiandrogen therapy before surgery, and in 78% (14/18) of those with hormone-resistant disease. However, Koeppen et al. [29] reported only five cases of HER-2 overexpression scores of + + in 61 patients with prostate cancer, with none scoring +++. The study by Lara et al. [30], of hormone-refractory prostate carcinoma, using biopsy specimens as part of the phase II trial of trastuzumab and docetaxel, reported that there was overexpression of HER-2 in 7% (of 100). The reasons for these contradictory results might lie in the differences between the techniques used to assess HER-2 overexpression, including differences in the clinical stages of the specimens being compared, the state of preservation of the antigens, the types of antibody used, and methods of restoring antigens. In addition, as the positive control
for the Hercep test was breast-cancer cell strains, further research might be needed to determine whether or not these results also apply to prostate cancer.

It is still unclear whether overexpression of the HER-2 oncprotein might serve as a prognostic factor for the outcome in patients with bone metastatic prostate cancer. The only retrospective investigation reported is that of Morote et al. [31] on c-erbB-2. They did not evaluate the time to recurrence, but reported that patients with overexpression had a poor prognosis, consistent with the present results.

In the present study, there was no correlation between HER-2 overexpression and NE cell differentiation. This suggests that NE cell differentiation is not induced by HER-2. However, Iwamura et al. [14] reported that the HER protein family might regulate NE cell growth. This disparity could be attributed to the tissues studied; by contrast with the cancerous tissue examined in the present study, they used normal prostate cells. Possibly HER-2 expression and NE cell differentiation are expressed independently. However, in the present study NE cell differentiation of the primary tumour did not reflect the outcome in patients with bone metastatic prostate cancer, and patients with HER-2 overexpression had a poor prognosis. These results might be regarded as a result of the more aggressive proliferation of tumour cells, because of the action of HER-2. Further research is needed on the mechanism whereby patients with HER-2 overexpression acquire resistance to endocrine therapy.

CONFLICT OF INTEREST

None declared.

REFERENCES

1 Abrahamsson PA. Neuroendocrine differentiation in prostate carcinoma. Prostate 1999; 39: 135–48
2 Bonkhoff H. Neuroendocrine cells in benign and malignant prostate tissue: morphogenesis, proliferation, and androgen receptor status. Prostate Suppl 1998; 8: 18–22
26 Nagakawa O, Fujiuchi Y, Fuse H, Saiki I. Differential effect of chromogranin A


Correspondence: Yoshiaki Yamada, Department of Urology, Aichi Medical University School of Medicine, Nagakute-cho, Aichi, 480–1195, Japan. e-mail: address: 101959@gk.amu.aichi-med-u.ac.jp

Abbreviations: NE, neuroendocrine; CgA, chromogranin A; HER-2, human epidermal growth factor-2; EOD, extent of disease; IHC, immunohistochemical.