Immunohistochemical Analysis of Neuroendocrine Differentiation in Prostate Cancer

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Key Words

Prostate cancer · Neuroendocrine cells · Calcitonin ·
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Abstract

Objective: To clarify the significance of neuroendocrine differentiation in prostate cancer. **Methods:** We immunohistochemically examined 96 samples of prostatic cancers obtained from radical prostatectomies using a specific neuroendocrine marker and various neuropeptides, as well as markers for cell proliferation, angiogenesis and androgen-receptor expression. **Results:** We frequently found neuroendocrine cells in atrophic glands with or without chronic inflammation in nontumorous tissues. Neuroendocrine cells were detected in 36.5% of prostate cancer samples overall, but had no significant correlation to angiogenesis, cell proliferation or biochemical recurrence. However, patients with a high frequency of neuroendocrine cells (9.4%) tended to undergo preoperative hormonal therapy ($p = 0.060$), which led to their cancers being atrophic with inflammation. The neuroendocrine cells in these patients contained calcitonin-positive cells ($p \leq 0.0001$), and calcitonin positivity showed significant association with high Gleason score ($p = 0.045$) in the group without preoperative therapy. In the group undergoing preoperative therapy, a high prostate-specific antigen titer at first admission correlated to significant biochemical

recurrence ($p = 0.003$). **Conclusions:** Neuroendocrine cells may be induced under conditions of atrophy with or without chronic inflammation in both noncancerous glands and in cancers, including calcitonin-positive cells. Neither positive neuroendocrine nor positive androgen-receptor status appears predictive for biochemical recurrence.

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Introduction

Neuroendocrine (NE) cells are normally sparsely distributed in the epithelia of many organs. Embryologically, most NE cells arise from the endoderm, rather than from the neural crest, and share a common stem cell with other epithelial cell types [1]. Dispersed NE cells are seen in the normal prostate, and contain neuropeptides including serotonin, somatostatin, calcitonin, bombesin, histamine, vasoactive intestinal peptide (VIP), α -human chorionic gonadotropin (α -HCG), parathyroid hormone-related protein (PTHrP) and cholecystokinin [1, 2]. Since receptors for some of these neuropeptides are also seen in normal prostatic epithelium [3, 4], it seems likely that NE cells and their products function in prostatic growth, differentiation, proliferation and modulation of surrounding target cells by intercellular paracrine or endocrine communication.

NE cells are detectable in prostate cancer (PC) patients using appropriate analytical methods [5]. In one in vitro study, androgen deprivation of the androgen-dependent LNCaP cell line induced NE differentiation [6], suggesting that, at least in PC, NE cells may differentiate from malignant epithelium, though other investigators dispute this theory [7]. However, it is well known that during hormonal therapy androgen-dependent cancers often become androgen-independent, giving rise to the hypothesis that NE cells induced by androgen deprivation secrete neuropeptides that stimulate proliferation of surrounding androgen-independent cancer cells. In the androgen-independent PC cell lines PC-3 and DU-145, neuropeptide receptors are expressed on the cytoplasmic membranes and exposure to calcitonin, neurotensin and/or PTHrP has been shown to promote cell proliferation in these lines [4, 8–11]. In a recent study, bombesin was apparently able to activate androgen receptor (AR) in PC cells under conditions of either low levels or total absence of androgen [12], suggesting that NE cells may induce AR activation via a ligand-independent pathway. Thus, depending on the analytical methods used, NE differentiation in PC may be an independent marker of poor prognosis [13–15] or an incidental and controversial phenomenon [16, 17].

In this study, we immunohistochemically investigated NE cells and their secretory role in relation to AR status in cancers of the prostate. Parameters such as cell proliferation, angiogenesis and stem or progenitor cell expression were also examined to further evaluate the biological behavior of NE cells in the prostate.

Materials and Methods

Tumor Samples

We examined a total of 96 PCs obtained from patients who had undergone radical prostatectomy. Of these patients, 46 had received preoperative hormonal therapy while 50 received no preoperative treatment. All tissue samples were fixed in 10% formalin for 48 h and trimmed at 5-mm intervals before processing through graded alcohols to paraffin. Paraffin blocks were then sectioned at 3- μ m intervals and stained with H&E for histological diagnosis. For each H&E sample, corresponding sections, to include cancer foci of interest, were serially cut at 4- μ m intervals for immunohistochemistry. The clinical and pathological features of 96 selected cases were compiled and are listed in table 1. The data collected included patient age, prostate-specific antigen (PSA) value, tumor Gleason score, tumor stage and biochemical recurrence (BR; defined by an increase in serum PSA value to >0.2 ng/ml after radical prostatectomy).

Table 1. Clinical and pathological features of the 96 cases

Preoperative hormonal therapy	46 (47.9%)
No preoperative therapy	50 (52.1%)
Mean age (range), years	69 (54–78)
PSA at first admission (range), ng/ml	22.8 $\pm$ 38.1 (4.0–288.0)
PSA at presurgery (range), ng/ml	7.5 $\pm$ 8.3 (0.0–50.3)
Gleason score	
5	5 (10.0%)/2 (4.3%)
6	20 (40.0%)/11 (23.9%)
7	21 (42.0%)/8 (17.4%)
8	1 (2.0%)/7 (15.2%)
9	2 (4.0%)/4 (8.7%)
10	1 (2.0%)/1 (2.2%)
NA	0 (0.0%)/13 (28.3%)
Stage (TNM staging system)	
I	24 (25.0%)
II	29 (30.2%)
III	25 (26.0%)
IV	12 (12.5%)
Unknown	6 (6.3%)
Capsular invasion	
+	36 (37.5%)
–	57 (59.4%)
Unknown	3 (3.13%)
Biochemical recurrence	
+	30 (31.3%)
–	51 (53.1%)
Unknown	15 (15.6%)

For Gleason score, the first figure refers to the score of prostatectomy specimens in the group without hormonal therapy, the second to the pretherapy score of biopsy specimens in the group with therapy. TNM = Tumor, node, metastases; NA = not applicable.

Immunohistochemistry

We used chromogranin A as a neuroendocrine-specific marker to detect NE cells. We divided the samples into 2 groups, classified as low (LNE) and high (HNE) frequency based on the relative numbers of NE cells detected within the tumors. Those samples which fitted the criteria of LNE consisted of PCs with single scattered NE cells (fig. 1a); those considered to be HNE consisted of PCs with NE cell clusters of at least 10 positive cells within a field at a magnification of $\times 200$ (fig. 1b).

We immunohistochemically stained PC samples using antibodies to 10 neuropeptides: bombesin, calcitonin, adrenocorticotrophic hormone (ACTH), glucagon, HCG, neurotensin, PTHrP, serotonin, thyroid-stimulating hormone (TSH) and VIP. At least 1 positive cell in the area of NE cells was regarded as showing the presence of a neuropeptide. Sections of HNE samples were also stained with antibodies to MIB-1 to assess cell proliferation, and with CD34 to evaluate angiogenesis. Based on their relative labeling indices, we subsequently divided MIB-1-positive cases into 3 groups: negative (0%), low (<5%) and high ($\geq 5\%$). In evaluating angiogenesis, we microscopically counted the capillaries around

cancer foci at 3 spots in a $\times 200$ field and recorded the mean number as a microvessel count. Randomly selected NE-negative PC cases were used as controls for MIB-1 and CD34.

Based on H&E evaluation, we selected for AR staining 19 of the 46 cases from patients exposed to hormonal therapy. These tumors were evaluated according to the sum of the percentages of cancer cells stained (1 = <25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%) and the intensity of nuclear staining in cancer cells (0 = absent, 1 = weak, 2 = moderate, and 3 = strong) [18]. Because a cancer stem cell theory has recently been proposed for many malignancies, including PC, we also examined the HNE cases for any association between the presence of stem cells or progenitor cells and NE differentiation using nestin, an intermediate filament protein. The procedure of immunohistochemical staining has been previously described [19]. The antibodies and their conditions are listed in table 2. The controls used were human fetal lung for bombesin, ileum for neurotensin, gastric antrum for VIP, thyroid C cells for calcitonin, pituitary glands for ACTH and TSH, pancreatic islets of Langerhans for glucagon, syncytiotrophoblasts of chorion for HCG and glioblastoma for nestin immunostaining; positive and negative controls were stained appropriately.

Statistics

Correlations among clinicopathological factors and NE cells, neuropeptides and AR were statistically examined using Mann-Whitney U analysis, Kruskal-Wallis analysis, χ^2 analysis and Fisher's exact test with StatView-J 5.0 software. We determined statistical significance to be $p < 0.05$ for all analyses.

Results

NE Cells in Nontumorous Tissues

We frequently found NE cells in the periurethral ducts and glands of seminal vesicles and in focal atrophic glands, with or without chronic inflammation marked by lymphocyte infiltration (fig. 1c, d). NE cells were not detected in hyperplastic and cystic dilated glands within the nodules of benign prostatic hyperplasia. These findings suggest that NE cells may be derived in nontumor-

ous tissues under certain conditions, such as glandular atrophy due to inflammation and/or hormonal therapy.

NE Cells in Prostate Cancers

Overall, we detected NE cells in 35 of the 96 PC cases, an incidence of 36.5%. NE cells were slightly less frequent in tumors exposed to preoperative hormonal therapy (15 of 46 cases, or 32.6%) and slightly more frequent in naive tumors (20 of 50 cases, or 40.0%).

There were 9 cases classified as HNE (9.4% of the total), which showed a relatively high frequency of NE cells within the individual tumors (fig. 1b). Their clinicopathological factors are summarized in table 3. Seven of those 9 cases had been exposed to preoperative therapy and showed atrophic glands with lymphocytic infiltration, and the NE cells were detected in a pattern similar to that seen in noncancerous tissues. All 9 HNE cases were negative for nestin expression (data not shown).

NE Cells and Neuropeptide Expression

We identified calcitonin expression in 37.1% of tumors (fig. 1e), neurotensin in 11.4% (fig. 1f), serotonin in 10% (fig. 1g), HCG in 8.6% (fig. 1h), VIP in 5.7% (fig. 1i) and bombesin in only 2.9% (fig. 1j) of the cancers we analyzed. ACTH, glucagon, PTHrP and TSH were not expressed in any of the cases. While the NE cells of HNE cases contained calcitonin-positive cells ($p \leq 0.0001$; fig. 1e; tables 3, 4), calcitonin positivity showed significant association with high Gleason score ($p = 0.045$) in the group without preoperative therapy.

Effect of NE Cells on Cell Proliferation and Angiogenesis

High MIB-1 indices were detected only in 2 (F189 and F312) of the 9 cases we classified as HNE (fig. 1k), the remaining 7 cases did show MIB-1 staining but with low indices (fig. 1l). Among our 9 randomly-selected control

Fig. 1. Immunohistochemical staining of chromogranin A (**a–d**), calcitonin (**e**), neurotensin (**f**), serotonin (**g**), HCG (**h**), VIP (**i**), bombesin (**j**), MIB-1 (**k, l**), CD34 (**m**) and AR (**n, o**). **a, b** NE cells in PC. **a** A single NE cell is recognized in a cancerous field (arrow) from the LNE group. **b** Several NE cells are clustered and easily found in a field from the HNE group. Magnification $\times 200$. The HNE cases tend to show atrophic changes in cancer glands, which are frequently accompanied by lymphocyte infiltration. **c, d** NE cells in noncancerous glands. NE cells are frequently found in focal atrophic glands with (**c**) and without (**d**) chronic inflammation. **e–j** Representative cases for calcitonin, neurotensin, serotonin, HCG, VIP and bombesin (arrow) stains in PC. **e** Serial sec-

tion of (**b**) as a representative case from the HNE group, which demonstrates positivity for calcitonin. **k, l** Representative cases for MIB-1 stain in PC. **k** The HNE group consists of the cases with $\geq 5\%$ positivity for MIB-1. **l** The LNE consists of the cases with $< 5\%$ positivity for MIB-1. A positive cell is shown by the arrow. **m** A representative case for CD34 stain in PC. Many capillaries are positive for CD34 around cancer glands. **n, o** Representative cases for AR stain in PC. **n** Case with high percentage of positive cells and strong intensity for AR (corresponding to categories 4 and 3). **o** Case with low percentage and weak intensity for AR (corresponding to categories 1 and 1; arrow). See Materials and Methods for an explanation of the categories.

Table 2. Antibodies used for detecting NE cells, neuropeptides and androgen receptor

Antibody	Source	Dilution	Antigen retrieval (autoclave)	Incubation time, min
Chromogranin A	Nichirei	1:1	not performed	60
Bombesin	Dako	1:500	performed	60
Calcitonin	Dako	1:1	performed	60
ACTH	Dako	1:1	performed	60
Glucagon	Dako	1:1	performed	60
HCG	Dako	1:1	performed	60
Neurotensin	SCB	1:50	performed	60
PTHrP	SCB	1:500	performed	60
Serotonin	Dako	1:1	performed	60
TSH (0042)	Dako	1:50	performed	60
VIP	SCB	1:100	performed	60
Androgen receptor	Zymed	1:1	performed	60
Nestin	Chemicon	1:200	performed	60

ACTH = Adrenocorticotrophic hormone; HCG = human chorionic gonadotropin; PTHrP = parathyroid hormone-related protein; TSH = thyroid-stimulating hormone; VIP = vasoactive intestinal peptide.

Table 3. Clinicopathological factors of 9 cases with HNE cells

Case number	Age	PSA 1 ng/ml	Therapy	PSA 2 ng/ml	GS	Stage	BR	Calcitonin	Serotonin	Neurotensin	VIP	HCG	Bombesin	CD34 MC	MIB-1	Glands
F171	62	53.6	treated	0.77	NA	pT3b	-	-	-	-	-	-	-	NA	NA	atrophic
F189	71	10.0	not treated	-	6	pT2b	-	+	-	-	-	-	-	26	H	nonatrophic
F192	67	34.7	treated	0.0	NA (7)	pT3b	-	+	-	-	+	-	-	61	L	atrophic
F235	69	4.2	not treated	-	6	pT3a	+	+	+	-	-	-	-	36	L	nonatrophic
F268	68	16.1	treated	0.0	NA (6)	pT2a	-	+	-	+	-	-	-	22	L	atrophic
F309	72	10.3	treated	-	NA (8)	pT2b	-	+	-	-	-	-	-	24	L	atrophic
F312	61	10.1	treated	1.6	NA (8)	pT2b	-	+	-	-	-	-	-	6	H	nonatrophic
F313	72	7.6	treated	-	NA (6)	pT2b	-	-	-	-	-	-	-	39	L	atrophic
F326	70	37.7	treated	2.2	NA	pT3b	+	-	-	-	-	-	-	18	L	nonatrophic

PSA 1 = PSA level at first admission; Therapy = preoperative hormonal therapy; PSA 2 = PSA level at presurgery; GS = Gleason score (biopsy score before therapy); BR = biochemical recurrence; MC = microvessel count; Glands = state of cancer glands; + = present; - = absent; H = high; L = low; NA = not available.

samples (tumors with no evidence of NE cells), we had 1 case with a high MIB-1 index and 8 cases with low indices. We were unable to detect any significant correlation between cell proliferation and the presence of NE cells or calcitonin expression (table 5).

As regards angiogenesis, we saw no significant correlation in CD34 immunopositivity with tissue NE cell density among the HNE and control groups. Under $\times 200$ magnification, the mean number of CD34-positive capillaries was 29 (range 6–61) in HNE cancers and 21 (range 10–36) in NE cell-negative tumors (fig. 1m).

Preoperative therapy did not significantly affect cell proliferation ($p = 0.464$) or angiogenesis ($p = 0.505$) in the HNE group.

Androgen Receptor in Prostate Cancers

Although the percentage of AR-positive cells and their staining intensities varied, all 19 cases receiving preoperative hormone therapy were positive for AR. In terms of specific percentages per staining category, 36.8% (7/19) of cases were scored as 1, 15.8% (3/19) scored as 2, 21.1% (4/19) scored as 3, and 26.3% (5/19) of cases were classi-

Table 4. Relationship between NE cells and clinicopathological factors

Factor	p value
NE (whole sample)	
Age	0.8632
PSA 1	0.5565
PSA 2	0.5652
Preoperative therapy	0.4523
Stage	0.1953
Biochemical recurrence	0.1705
HNE (whole sample)	
Age	0.2341
PSA 1	0.7610
PSA 2	0.0465*
Preoperative therapy	0.0596
Gleason score	0.27
Stage	0.1744
Biochemical recurrence	0.8072
Calcitonin	<0.0001*
HNE (preoperative therapy)	
Age	0.0982
PSA 1	0.5892
PSA 2	0.8268
Gleason score	0.74
Stage	0.1861
Biochemical recurrence	>0.9999
Androgen receptor (percentage)	0.6129
BR (preoperative therapy)	
PSA 1	0.0348*
PSA 2	0.5228
Stage	0.4895
HNE (no preoperative therapy)	
Age	0.9580
PSA 1	0.1022
Gleason score	0.68
Stage	0.8104
Biochemical recurrence	0.8258
BR (no preoperative therapy)	
PSA 1	0.3316
Stage	0.4124

* Significant p value. NE = All cases with NE cells; PSA 1 = PSA level at first admission; PSA 2 = PSA level at presurgery.

fied as 4. When defined according to the degree of nuclear staining intensity, 0 (0%) were in group 0, 9 (47.4%) were in group 1, 3 (15.8%) were in group 2 and 7 (36.8%) were in group 3. Representative cases with a high staining percentage and strong staining intensity (corresponding to percentage category 4 and intensity category 3) and a low percentage and weak intensity (corresponding to per-

Table 5. Biological factors of NE cells and calcitonin

Factor	p value
HNE	
CD34, microvessel count	0.2472
MIB-1	0.9293
Calcitonin	
CD34, microvessel count	0.4795
MIB-1	0.0865

centage category 1 and intensity category 1) are illustrated in figure 1n and o.

Analysis of Clinicopathology and NE Cell Frequency

We analyzed clinicopathological factors for the presence and relative numbers of NE cells within tumors to see if there were any functional correlations; these are summarized in table 4. The Gleason scores of biopsy specimens obtained prior to the start of preoperative hormone therapy are substituted for Gleason scores assigned after tumor excision so that comparisons could be made with scores for untreated tumors. HNE cases showed no significant correlation with Gleason score, tumor stage, BR or AR expression. AR expression alone did not significantly correlate with Gleason score, tumor stage or BR. However, low presurgical PSA values were significantly associated with HNE cell populations ($p = 0.047$) and high Gleason scores significantly correlated with both high presurgical PSA levels ($p = 0.021$) and high tumor staging ($p = 0.034$). High PSA levels at first admission were significantly associated with BR only in patients undergoing preoperative hormone therapy ($p = 0.0348$). High Gleason scores were also significantly correlated with high tumor stage ($p = 0.027$) and with the presence of capsular invasion ($p = 0.047$).

Discussion

The function of NE cells and the method of their differentiation in PCs are still unclear. In noncancerous tissue, NE cells were not found in hyperplastic prostate tissue, but they tended to be present in atrophic glands resulting from certain conditions, such as inflammation and/or hormonal therapy. The finding that NE cells are more distributed in atrophic glands has been reported previously [1], which may provide a clue to NE differentiation. NE cells contain various neuropeptides

that may stimulate proliferation of surrounding cells. De Marzo et al. [20] proposed a model of proliferative inflammatory atrophy (PIA) as a precancerous lesion, in contrast to prostatic atrophy with inflammation. PIA is highly immunopositive for MIB-1 [21], and there appears to be a transition from PIA to high-grade prostatic intraepithelial neoplasia-like lesions [22]. The higher than expected numbers of NE cells we detected in our samples of noncancerous atrophic glands with inflammation may have been due to inadvertent inclusion of some PIA tissues.

While some studies have found NE cells in up to 100% of PCs surveyed [5], we detected NE cells in 36.5% of our PC samples overall. Only 9.4% of PC cases (9/96) were categorized as HNE in our study, but even within this group, NE cells tended to be found more often and more densely in chronically inflamed atrophic glands resulting from preoperative hormonal therapy and without significant correlation to proliferative activity, as indicated by the relatively low levels of MIB-1 immunostaining. This suggests that NE cells may be induced in areas of PC that are biologically similar to noncancerous atrophic glands. It may be that NE differentiation is induced by certain conditions causing glandular atrophy, notably by cytokines released by lymphocytes in cases of chronic inflammation. Paracrine secretion of neuropeptides by NE cells possibly functions to enable the glands only to recover from insult, or the proliferative effect of neuropeptides may be gentler in vivo. Interleukin-6 and interferon- γ are mentioned as candidates for cytokines, which may induce NE differentiation [23, 24].

The expression of nestin, a stem cell-associated intermediate filament, is now known to characterize cancer cells and stem or progenitor cells in a variety of tumor lineages. Associations of nestin expression, androgen withdrawal and metastatic potential have been confirmed in PCs [25]. Based on this claim, PC treated with hormonal therapy is expected to include nestin-positive cells. However, all 9 of our HNE cases were completely negative. This may be due to the fact that these 9 HNE samples were not from clinically metastatic disease and we may have been observing an early stage of NE differentiation.

The most commonly identified neuropeptide in our tumor samples was calcitonin, which was detected in 37.1% of the cases. This was followed by neurotensin in 11.4% of tumors, serotonin in 10%, HCG in 8.6%, VIP in 5.7% and bombesin in 2.9%. We did not detect ACTH, glucagon, PTHrP or TSH in any sample. This is in marked contrast to previously reported PC neuropeptide profiles

that have shown 5–8% of tumors to be positive for calcitonin, 0% for neurotensin, 0–54% for serotonin, 22% for HCG, 4% for bombesin, 0 to <5% for ACTH, 0% for glucagon, 100% for PTHrP and 0% for TSH [26–28]. We could find no report for the frequency of VIP in PC. It is noteworthy that the NE cells of HNE cases contain calcitonin-positive cells ($p \leq 0.0001$). Calcitonin induces proliferation in vitro in the androgen-independent PC-3 and DU-145 cancer cell lines [8, 29], and is correlated with angiogenesis in vivo through its effect on endothelial cells [30], resulting in tumor growth. Regrettably, calcitonin-positive HNE cases were not significantly associated with an increased proliferation rate in surrounding tissues or with stromal angiogenesis in this study. This may have been due to experimental conditions such as the small number of HNE cases and the limited area examined. Nonsignificant results may have occurred as a result of the complexity of the in vivo environment, for example, the time interval from calcitonin secretion to react with the surrounding cells or the inappropriate concentration of calcitonin secreted. Further study may be required. However, in the tumors from patients with no preoperative therapy, the presence of calcitonin was significantly associated with a high Gleason score ($p = 0.045$), suggesting a relation to cancer aggressiveness or infiltration. In a spheroid disaggregation/migration assay, calcitonin significantly enhanced disaggregation/migration of PC-3M spheroids, altered the surface activity of adhesion molecules, and stimulated the secretion of gelatinases (MMP-2 and MMP-9) from PC-3M cells [31, 32].

We found AR expression in all cases treated preoperatively with hormonal therapy but we did not find any significant correlation between AR expression, the presence of NE cells or BR. This expression pattern of AR in relation to NE differentiation is in line with previous observations in PC [33], and these facts suggest that neuropeptides from NE cells do not affect the AR expression status of surrounding cancer cells. After androgen-deprivation therapy, AR-positive cancer cells are not predictive of the time to progression [34], suggesting that activation of AR signaling is directly caused by neuropeptides from NE cells independently of the AR expression status.

In examining the relationships between clinicopathological parameters, we were able to detect significant positive associations only for PSA levels and Gleason scores: high presurgical PSA titers were predictive of tumors with both high Gleason ratings and pathology grade. Elevated PSA levels prior to preoperative androgen ablation were significantly correlated to tumor recurrence. Low

presurgical PSA values were significantly associated with high tumor NE cell counts in a subset of lesions. Although these HNE tumors represented a small percentage (9.4%) of the total number of tumors in this study, most of these tumors (77.8%) were from patients who had undergone preoperative hormone therapy, and the higher frequency of NE cells is probably related to the therapy-induced glandular atrophy with or without inflammation, which appears to promote their differentiation.

In summary, we believe that NE cells may be induced under conditions of atrophy with or without chronic inflammation in both noncancerous glands and in cancers

exposed to androgen-deprivation therapy, including calcitonin-positive cells, which may affect surrounding cells independently of the AR expression status. However, neither positive NE nor positive AR status appears predictive for biological tumor recurrence in prostatic cancers.

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