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Comparative analysis of biological profiles of benign prostate hyperplasia and prostate cancer as potential diagnostic, prognostic and predictive indicators

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Abstract: The prognosis in prostate cancer depends on several clinical-morphological factors, such as Gleason score, pTNM and preoperative PSA level. Reliable biological markers are being sought to supplement clinical-morphological data in order to better predict prognosis and to select an individualized therapeutic option. The aim of this study was a comparative analysis of the expression of biological markers, such as Hif-1α, bcl-2, p53, Ki-67, cyclin D1 and CD44 in BPH and prostate cancer, as well as examining their association with standard prognostic factors in prostate cancer. The immunohistochemical analysis was made on 82 formalin-fixed, paraffin-embedded tissue blocks: 43 prostate cancer specimens derived from patients who had undergone radical resection, and 39 prostate bioptates derived from patients with BPH. A positive correlation was demonstrated between Gleason score and the expression of both Hif-1α ($R = 0.32$, $p < 0.05$) and Ki-67 ($R = 0.30$, $p < 0.05$). Additionally, a negative correlation was demonstrated between tumor stage (pTNM) and bcl-2 expression ($R = –0.35$, $p < 0.05$). Hif-1α as a hypoxia marker and Ki-67 as a proliferation marker, both correlated with Gleason score, may constitute important additional prognostic indicators in prostate cancer patients. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 3, pp. 452–457*)

Key words: prostate cancer, benign prostate hyperplasia, biomarkers

Introduction

The search for biological markers of carcinogenesis is one of the most important fields of contemporary studies aimed at detecting and treating prostate cancer. These biological markers are expected to supplement (or even partly replace) information on standard prognostic and predictive indicators, such as preoperative PSA level, Gleason score and clinical staging. The most reliable and decisive biomarkers of an individual risk of disease progression and a chance of successful treatment could also indicate differences in biological profiles of both benign and malignant lesions, which would be helpful in their differential diagnosis.

In order to make a comparative evaluation of processes known to be crucial for carcinogenesis, such as proliferation, hypoxia, apoptosis and cellular adhesion, we conducted an immunohistochemical staining of their representative proteins in material derived from patients with prostate cancer and benign prostate hyperplasia (BPH).

The proliferation rate, a feature that has been proven to be an unfavorable prognostic factor in many cancers, was evaluated using its two widely known indicators: Ki-67 (Mib-1) protein, which is expressed by proliferating cells during all active phases of the cell cycle, and cyclin D1, which is involved in controlling the G1/S phase [1–3].

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The contribution of hypoxia, a factor proven to increase radioresistance and limit the efficacy of chemotherapy, was estimated on the basis of the staining of its endogenous marker: hypoxia-inducible factor 1α (Hif-α) [4–6]. The protein is a central mediator of a cellular response to hypoxic conditions. It promotes transcription of genes which are essential for tumor progression and are responsible for both neoplastic angiogenesis and local infiltration [7, 8].

Apoptosis was evaluated based on the staining of two proteins: bcl-2 and p53. Bcl-2 is a factor responsible for apoptosis inhibition [9], while p53 is a key factor activating apoptosis in cases of unrepairable DNA injury as a result of a broad-spectrum of stressful agents (e.g. hypoxia or oncogene expression) [10].

Cellular adhesion, a feature connected with tumor progression and an ability for metastasizing [11] was studied using CD44 protein staining. CD44 glycoprotein performs the function of a cellular surface receptor for hyaluronic acid, a glycoaminoglycoside which takes part in cancer cell metastasizing [12]. Three forms of CD44 have been identified: full length (exons 1–20), standard (exons 1–5, 16–20) and CD44v [13].

Material and methods

Patients. A retrospective immunohistochemical analysis comprised 82 formalin-fixed, paraffin-embedded tissue blocks derived from 43 prostate cancer patients who had undergone radical resection and 39 prostate bioptates derived from patients with BPH. The average age of prostate cancer patients on the day of the operation was 64.4 ± 6.4 years (range 48–75 years), and of BPH patients it was 66.6 ± ± 7.1 years (range 51–80 years). The average preoperative PSA level was 11.1 ± 7.0 ng/ml (range 3.31–39.90 ng/ml) and 8.4 ± 4.4 ng/ml (range 1.0–21.3 ng/ml), respectively in prostate cancer and BPH patients. The average Gleason score was 6.2 ± 1.3 (range 3–9). The post-operative clinical stage was T2N0M0 (pT2N0M0) in 21 patients and pT3N0M0 in 22 patients.

Immunohistochemistry. The analysis was conducted with the EnVision method using the EnVision+System HRP (horseradish peroxidase, K 4001 and K 4002, Dako) kit and adequate monoclonal antibodies. 5 μm-thick sections derived from 10% formalin-fixed, paraffin-embedded tumors were placed onto basic adhesive slides and incubated for two hours at 60°C in a chamber thermostat. Then the sections were deparaffinized in xylene and rehydrated in decreasing ethanol dilutions. Endogenous enzymatic activity of peroxidase was inhibited with 3% hydrogen peroxide solution. Depending on which antibody, a microwave oven or a water bath was used for the epitope retrieval. The first method was used for such mouse monoclonal antibodies as: anti-Hif-1α (clone H1 alpha67, Chemicon International), anti-p53 (clone DO-7, Dako), anti-bcl-2 (clone 124, Dako), anti-Ki-67 (clone MIB-1, Dako) as well as rabbit monoclonal anti-cyclin D1 (clone SP4, Lab Vision). The second method was used for mouse monoclonal anti-CD44 (clone DF1485-CD44s, Dako). Then the sections were incubated with primary antibodies (1:100 for Hif-1α, p53, bcl-2 and Ki-67; 1:50 for cyclin D1 and CD44), and afterwards with the EnVision+System HRP reagent. Chromogen DAB (3,3 diaminobenzidine, K3468, Dako) was used to demonstrate the examined cellular structures. Cell nuclei were stained with hematoxylin (S 2020, Dako). As the last step, the sections were hydrated in increasing ethanol dilutions, cleared in xylene and mounted in medium (Consul Mount, Thermo Shandon, Runcorn, UK).

Biomarkers assessment. All slides were reviewed by two researchers (E.Ś. and H.A.) using a light microscope with a micrometric insertion (Olympus, Poland). The viewing fields were evaluated at 40-fold magnification of an objective lens. At least 500 (max. 1,000) cells of prostate cancer and at least 300 BPH cells were counted in several (7–10) viewing fields [14], which were selected randomly due to the homogenous distribution of all studied staining products in both cancer and BPH specimens. The results of nuclear (Hif-1α, p53, Ki-67, cyclin D1), cytoplasmic (bcl-2) and membrane (CD44) staining were shown in the form of labeling indices (LI) interpreted as a percentage of positively stained cells among a total number of examined cells. Additionally, in case of nuclear staining of prostate cancer specimens for HIF-1α, cyclinD1 and p53, the intensity of immunohistochemical reaction and the percentage of the most intensely stained nuclei were evaluated based on the Remmele et al. method [15] (Table 1). The final result (reaction intensity) was presented as a product of these two parameters (0–12) according to the scale: 0–2 = negative or low, 3–6 = moderate and > 6 = high [16].

Statistics. An accordance of the variables distribution with a normal distribution was verified with the Shapiro–Wilk test. In case of samples of approximately normal distribution (patients' age) the *t*-student test was used to compare

Table 1. Evaluation of the immunohistochemical reaction intensity acc. to the Remmele et al. method [15]

Reaction intensity	Percentage of the stained nuclei
0 — no reaction	0 — no reaction
1 — low intensity reaction	1 — reaction in < 10% of cells
2 — moderate intensity reaction	2 — reaction in 10–50% of cells
3 — high intensity reaction	3 — reaction in 51–80% of cells
	4 — reaction in > 80% of cells

mean values of the independent variables. In other cases (biomarkers) a difference between the groups was analyzed with the Mann–Whitney U test. The correlation between the pairs of parameters was described by the Spearman's correlation coefficient. The p value ≤ 0.05 was accepted as the limit of statistical significance. The calculations were performed using the commercial software package (Statistica, StatSoft, Tulsa, OK, USA).

Results

A positive nuclear staining of Hif-1α was observed in 90.7% (39/43) of the tumors and in 12.8% (5/39) of BPH. The level of hypoxia in the prostate cancer group was significantly higher than in the BPH group (LI: 16.1 ± 13.8 and 0.61 ± 2.55, respectively; $p < 0.001$) (Figures 1A1, A2). A cytoplasmic Hif-1α staining was also occasionally observed but was not considered in the analysis. The low intensity reaction (1–2) was observed in 17 tumors (39.6%) and moderate intensity reaction (3–6) in 22 tumors (51.1%). The high intensity reaction was not observed. For the BPH group, we did not present results based on the Remmele method because a positive nuclear staining was observed in only 12.8% of cases and the reaction intensity was very low. A positive correlation was demonstrated between Hif-1α labeling index and Gleason score ($R = 0.32$, $p < 0.05$; Figure 2). There was no correlation between Hif-1α labeling index and the other standard prognostic factors (i.e. PSA, pTNM).

A positive cytoplasmic reaction with anti-bcl-2 was achieved in 39.5% of prostate cancers (17/43) and in 94.9% of BPH (37/39). Bcl-2 expression was significantly higher among patients with BPH compared to those with cancers (LI: 6.97 ± 16.42 and 26.21 ± 18.89, respectively; $p < 0.001$) (Figures 1C1, C2). In the prostate cancer group, there was a negative correlation between bcl-2 expression and pTNM ($R = –0.35$; $p < 0.05$; Figure 2).

A nuclear reaction with anti-p53 was observed in 69.8% of prostate cancers (30/43) and in 74.4% of BPH (29/39). There was no significant difference of p53 expression between the groups (LI: 11.86 ± 19.51 and 2.03 ± 2.27, respectively; $p > 0.05$) (Figures 1F1, F2). For most tumors (27/43), the expression intensity was weak or moderate (2–6). An intense expression (9) was observed in three cases and it comprised over 60% of cancer cells. For the BPH group, we did not present results based on the Remmele method because a positive nuclear staining was observed in a small number of cells and the reaction intensity was very low. No significant correlation was proven between p53 LI and the standard prognostic factors of prostate cancer.

Ki-67 expression was observed in 93% (40/43) of the tumors and in all BPH cases, while a nuclear reaction for cyclin D1 was reported in 93% of prostate cancers (40/43) and in 64.1% of BPH (25/39). Ki-67 expression for prostate cancers appeared to be higher than for BPH (Ki-67 LI: 6.13 ± 5.41 vs. 1.44 ±

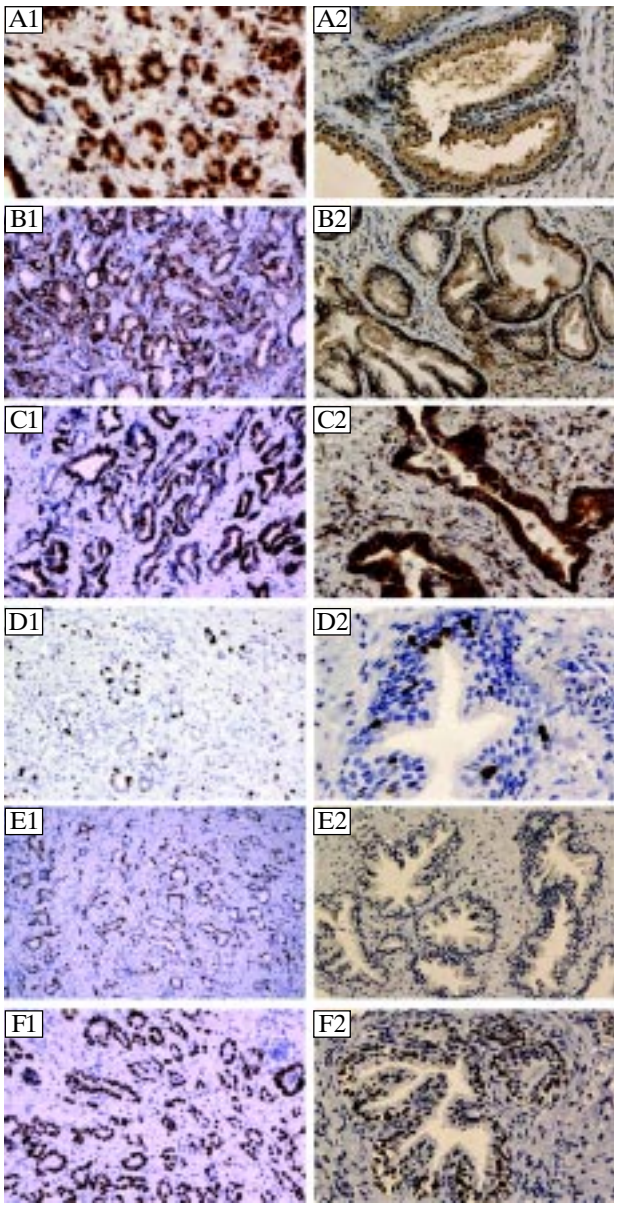

Figure 1. Immunohistochemical staining of prostate cancer and BPH. Nuclear: Hif-1α: **A1** (LI: 16.1 ± 13.8), **A2** (LI: 0.61 ± 2.55); Ki-67: **D1** (LI: 6.13 ± 5.41), **D2** (LI: 1.44 ± 0.99); Cyclin D1: **E1** (LI: 9.89 ± 9.73), **E2** (LI: 5.31 ± 6.58); p53: **F1** (LI: 11.86 ± 19.51), **F2** (LI: 2.03 ± 2.27). Cytoplasmic and membrane: CD44: **B1** (LI: 12.77 ± 16.52), **B2** (LI: 3.94 ± 4.45). Cytoplasmic: bcl-2: **C1** (LI: 6.97 ± 16.42), **C2** (LI: 26.21 ± 18.89). Original magnification: × 100 (**E1**); × 200 (**A2**, **B1**, **B2**, **C1**, **D1**, **E2**, **F1**); × 400 (**A1**, **C2**, **D2**, **F2**). Lower magnification in the case of prostate cancer was chosen to better visualize a higher percentage of positive immunohistochemical reaction in cancer cells compared to BPH cells. Higher magnification in the case of BPH was chosen to better visualize the type of immunohistochemical reaction

± 0.99, p < 0,001; cyclin D1 LI: 9.89 ± 9.73 vs. 5.31 ± ± 6.58, p < 0.01) (Figures 1D1, D2, E1, E2).

In the case of cyclin D1, a low intensity reaction (1–2) was observed in 25 tumors (58.1%) and moderate intensity reaction (3–6) in 15 tumors (34.9%). We did not observe any high intensity reaction. For the BPH group, we did not present results based on the Remmele method because a positive nuclear staining was observed in a small number of cells and the reaction intensity was very low.

A positive correlation between Ki-67 labeling index and Gleason score was noted (R = 0.30, p < 0.05; Figure 2). In the BPH group, there was a significant increase in Ki-67 LI with an increasing patient age (R = 0.34, p < 0.05).

No correlation between cyclin D1 LI and the standard prognostic factors of prostate cancer was shown.

The percentage of tumors with a membrane or cytoplasmic reaction for a presence of CD44 protein was 86% (37/43). CD44 expression in the BPH group was reported in 76.9% (30/39) of cases. The difference between the prostate cancer and BPH groups was significant (LI: 12.77 ± 16.52 and 3.94 ± 4.45, respectively; p < 0.01) (Figures 1B1, B2). There was no correlation between CD44 LI and the standard prognostic factors of prostate cancer.

Discussion

The literature regarding the biological factors affecting a pathophysiological picture of both BPH and prostate cancer is equivocal, and often contradictory.

Our study proved a significant difference in the expression of the studied biological markers, including Hif-1α, Ki-67, cyclin D1, CD44 (with the exception of p53), between BPH and prostate cancer.

Interestingly, at the same time, we did not observe any significant difference in the preoperative PSA level between the analyzed groups of patients. Additionally, PSA was the only standard prognostic factor that did not show any relationship with the examined biological parameters. Our findings define PSA as a factor which is not correlated with prostate cancer biology, but only with a presence of any pathological process in the prostate. As an organ-specific marker, it still remains a valuable indicator of processes proceeding with prostate architecture disturbances, including benign nodular hyperplasia and prostatitis. Simultaneously, PSA is still the only broadly clinically exploited monitor of the post-treatment course of prostate cancer [17].

The most significant differences of biomarkers expression between the study groups were regarding Hif-1α and Ki-67 (p < 0.001), which indicates a significant role of hypoxia and cellular proliferation in

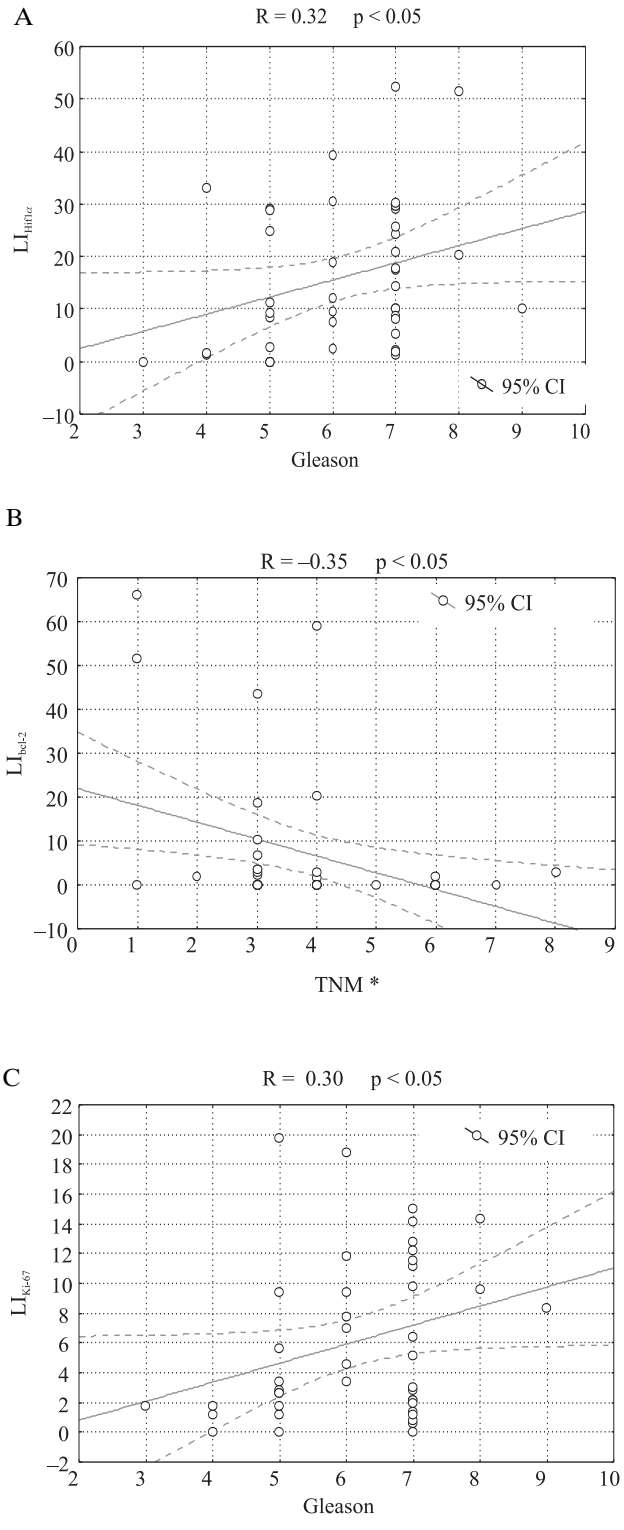

Figure 2. A. Positive correlation between Gleason score and Hif-1α LI (R = 0.32, p < 0.05). **B.** Negative correlation between pTNM and bcl-2 LI (R = –0.35, p < 0.05; pTNM groups correspond to: 1 — pT2aN0M0; 2 — pT2bN0M0; 3 — pT2cN0M0; 4 — pT3aN0M0; 5 — pT3bN0M0; 6 — pT3cN0M0; 7 — pT3bN1miM0; 8 — pT3cN1M0). **C.** Positive correlation between Gleason score and Ki-67 LI (R = 0.30, p < 0.05)
CI — confidence interval

carcinogenesis and prostate cancer progression. The correlation between the expression of these two proteins and Gleason score might confirm this conclusion. An increasing proliferation rate seems to promote both a lower differentiation degree of cancer cells and a higher hypoxia degree of a growing tumor, not sufficiently supplied with oxygen and nutrients by a pathological net of vessels.

The literature data on a relationship between hypoxia and standard prognostic factors in prostate cancer is equivocal. Carnell et al. confirmed our results [18], while Du et al. reported no correlation between Hif-1α and clinical parameters [19]. Interchangeably however, a high proliferative index in prostate cancer has been reported to be a factor associated with radiotherapy failure, a higher risk of metastases and a shorter recurrence-free survival [20, 21]. Interestingly, our study showed a significant increase of Ki-67 expression with an increasing patient age in the BPH group, suggesting a possibility of transformation from BPH to prostate cancer through an escalation of the proliferation process. We also observed significantly higher cyclin D1 labeling indices in the prostate cancer group, although no correlation between the protein expression and the standard prognostic factors was shown. Our observation is consistent with the results of Aaltomaa et al. [22], but contrary to some other authors' reports of a relationship between cyclin D1 expression and both low preoperative PSA level and high Gleason score [23]. The lack of high intensity nuclear reaction and relatively low labeling index for both cyclin D1 (meaning cells in the G1/S phase) and Ki-67, might reflect a slow growth of the studied tumors (none of them metastasized).

Our results suggest that cyclin D1 has a limited value as a potential biomarker supporting the choice of a therapeutic option, since it seems to be mainly expressed in advanced prostate cancers. Our findings are consistent with Drobnjak et al. [24], who found a higher cyclin D1 expression in prostate cancer patients with bone metastases compared to those without cancer dissemination.

The results of bcl-2 and p53 staining did not give an unequivocal description of the apoptosis process in either BPH or prostate cancer. We noted a significantly higher bcl-2 expression in the BPH group compared to the cancer group. The negative correlation of the protein expression with pTNM might suggest its meaningful role in the early stage of the cancer. Additionally, since there is a proven relationship between apoptosis and hypoxia, we cannot exclude that an increasing (with tumor growth) hypoxia correlates with a decreasing inhibition of apoptosis. Our findings are contrary to the results of Bubendorf et al. [25], who reported a high bcl-2 level in advanced prostate cancer, and consistent with the observations of Tolonen et al. [26] of a higher bcl-2 expression in PIN (prostatic interepithelial neoplasia) than in prostate cancer. Additionally, we noted an increase in the level of cyclin D1 with a decrease of bcl-2 expression ($R = -0.32$, $p < 0.05$), which suggests a modulation of cyclin D1 synthesis by bcl-2 protein and is consistent with other authors' results [27]. The results on the other apoptosis-connected protein, p53, which did not show any relationship with the standard prognostic factors or any difference of expression between the groups, suggest that the p53 tumor suppressor gene mutations do not influence a differentiation degree or clinical stage of prostate cancer. Our findings confirmed the reports of Karaburun et al. [28]. But they contradict the observations of Fonseca et al. [29] of a positive correlation between p53 expression and both an increasing PSA level and clinical stage. We also noted a significant positive correlation between p53 labeling index and Ki-67 labeling index ($R = 0.35$, $p < 0.005$), confirming that the mutated gene p53 protein loses the ability to inhibit cell proliferation. Our data is consistent with the results of Amirghofran et al. [30] and Bergera et al. [31].

Cellular adhesion proteins, like CD44, belong to the group of promising biomarkers and have been widely analyzed in different types of cancers [32]. Although we showed a significant difference of CD44 immunoreactivity between the BPH and prostate cancer groups, no correlation between the protein expression and the standard prostate cancer prognostic indicators was found. Our findings do not confirm the results of Aaltomaa et al. [33], who observed a negative correlation between CD44 expression and Gleason score, preoperative PSA level, and clinical stage.

We conclude that Ki-67 as a marker of proliferation, and Hif-1α as a hypoxia marker, are the most reliable biological markers of prostate cancer and may be useful in both differentiating from BPH and predicting answer for treatment (e.g. better outcome after high fraction doses of irradiation in cases of highly hypoxic tumors) as well as prognosis (e.g. higher probability of recurrence in cases of fast proliferation or high level of hypoxia). Because they are easily estimated by immunohistochemical methods, they may be applicable in everyday practice as an important additional clinical tool.

Conflict of interest

The authors declare no conflict of interest.

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