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PROGNOSTIC VALUE OF BIOLOGICAL MARKERS IN SUPERFICIAL BLADDER TUMORS

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SUMMARY

• *The early identification of urinary bladder cancer patients, who are at a high risk of developing recurrences and tumor progression, is of utmost importance, because they require a more aggressive therapeutic approach and close follow-up. The routine use ofnewprognosticators, more specific and reliable than the established clinicopathologic factors, is therefore mandatory. The present work reviews some of the most significant biological tumor markers (cell cycle proteins, blood group antigens, tumor suppressor genes, oncogenes, CD44 gene transcripts, etc.) defined immunohistochemically in bladder cancer patients. It summarises most of the recently published results of the clinical trials, using these markers as predictors of the tumor growth, invasion and metastasis. It is also an attempt to highlight the present state of the problem, the still existing controversy and some of the hypotheses concerning the predictive potential of the recently detected tumor markers.*

INTRODUCTION

• Bladder cancer is the second most prevalent urological malignancy in men, accounting annually for approximately 10 000 deaths in USA (1). Of all newly diagnosed cases 70-85% are superficial in stage and so potentially completely curable. However, 50-80% of all superficial bladder tumors (SET) recur and 25-30% of these recurrences show a higher stage of malignancy or metastases (2).

The early identification of patients, who are at a high risk of developing recurrences and tumor progression, is of utmost importance, because they require a more aggressive therapeutic approach, including early total cystectomy. The prognostic value of many clinicopathological tumor parameters (stage, grade, type, size, multiplicity, location, etc.) has been well documented. The results from the quadrant, "mapping", cold-cup biopsies, detecting the presence of associated carcinoma *in situ* in normally looking mucosa, provide additional prognostic information. The quantitative morphometric methods, flow cytometry and image analysis, both of which have been rapidly introduced in the clinical practice in the last few years, seem to be helpful in this aspect. All these prognostic factors, either taken alone or in various combinations, appear to be, however, insufficient in predicting the tumor behavior in the individual patient (3). They provide only a general guide for clinical management and none of them gives at present reliable information which specifically correlates with the prognosis of an individual tumor. Therefore, the routine use of new, more specific and effective prognosticators is necessary. This need justifies the clinical application of immunohistochemical methods, defining biological tumor markers with a strong predictive value.

My main objective here is to review recent papers discussing the prognostic role of the most significant biological markers for predicting recurrences, tumor progression and patient survival in SET.

In our summing up of the literature, nine main groups of biological tumor markers were clearly distinguished. They will be reviewed in the same order as they are presented in Table 1 (see also Table 2 for some abbreviations used in evaluating tumor stage and grade).

Table 1. *Biological market's in bladder tumors*

Cell cycle markers
Tumor-associated antigens
Blood group antigens
Growth factors
Enzyme activity
Biochemical parameters
Oncogenes and tumor suppressor genes
Genetic abnormalities
CD44 gene transcripts

Table 2. Tumor stage and grade according to the classification of the International Union against Cancer

Stage/grade	Abbreviations
pTis	pre-invasive carcinoma (carcinoma *in situ*)
pTa	papillary non-invasive cancer
pT1	tumor not extending beyond the lamina propria
pT2	tumor invading superficial smooth muscle
pT3	tumor invading deep muscle
G1	high degree of differentiation
G2	medium degree of differentiation
G3	low degree of differentiation

CELL CYCLE MARKERS

- **Bromodeoxyuridine test**

Bromodeoxyuridine (BrdU), applied intravenously 3 to 6 h prior to transurethral resection of bladder tumors, maybe used as an *in vivo* test for determining the fraction of tumor cells in S phase of the cell cycle (4). The mean labelling index correlated with the tumor stage and grade. Moreover, the SBT that recurred during the follow-up period had a mean labelling index (at diagnosis) of 8.4 %, whereas the tumors that did not recur had a mean labelling index of 3.4 %. These data suggest that the *in vivo* determination of BrdU uptake in SBT may be useful in predicting which tumors are likely to recur following resection and so rationalising both the time to follow-up and intravesical chemoprophylaxis.

BrdU labelling cell index status was the third in significance prognostic factor in the study of Tachibana *etal* (5). Patients with low BrdU labelling cell index rumors demonstrated a 98% 3-year survival rate, compared to 42.9 % for those with high-grade **tumors.**

- **Proliferating cell nuclear antigen**

Proliferating cell nuclear antigen (PCNA) is a protein with a molecular weight of 36 kD. It is detected in the proliferating cells and serves as a marker of the tumor growth. The fraction of PCNA positive nuclei is related to a variety of the known prognostic factors, such as stage, grade, and DNA ploidy (6,7). In pTa-pTl tumors, the fraction of PCNA-positive nuclei predicts tumor progression and patient survival. In a multivariate survival analysis, PCNA-positive nuclei show independent predictive value and may be used as a significant prognostic variable of patient outcome. Patients with PCNA indices above the median level (12 %) have shown a worse prognosis. The measurement of PCNA labelling index in bladder cancer may thus prove to be an objective and quantitative assay of biological aggressiveness and may provide significant prognostic information.

- **Ki67 antigen**

The monoclonal antibody Ki67 recognises a cell cycle-related nuclear antigen revealed in actively dividing cells. Ki67 reactivity has been shown to correlate with conventional prognostic indicators, such as tumor stage and grade, in bladder cancer patients (8). Limas *et al* (9) evaluated the proliferation activity of urothelial neoplasms, comparing two proliferation indices: the percentage of nuclei labelled by BrdU (BrdU index) and the percentage of nuclei expressing Ki67 antigen (Ki67 index). These were very low in the urothelium of the normal controls. Non-invasive SBT had increased BrdU and Ki67 indices, but both were below those of the invasive tumors. In addition, the number of Ki67-positive cells in G2 SBT might be an useful aid in separating those with a favourable prognosis from those with a poor clinical outcome (10). These authors also found a significant difference in Ki67 score between pTaG2 and pT!G2 SBT.

TUMOR-ASSOCIATED ANTIGENS

• Tumor-associated markers defined by monoclonal antibodies have proven useful to phenotype bladder tumors. Us-

ing multiparameter flow cytometry to analyse simultaneously DNA content and the expression of surface glycoproteins defined by monoclonal antibodies T16, Tm5, T43 and T138 (11), it was found that T138 antigen expression is a better single indicator of cancer progression and patient survival than was DNAploidy status (12). The results suggest that simultaneous flow cytometry measurements of DNA and surface antigens may better assess the malignant course of human bladder tumors.

Monoclonal antibodies (10D1,7C12,6D1,3C6,C4 andE7) directed against bladder tumor cells were tested using flow cytometry (13). Analysis of DNA content revealed two groups of patients, with unimodal andbimodal profiles. All cells from Gl tumors (from the first group) were labelled with 10D1 and 6D1 antibodies. G3 tumor cells were labelled with C4 and E7 antibodies and most of them were with a bimodal DNA profile.

- **CA-50 antigen**

Monoclonal CA-50 antibody is an useful tumor marker in gastrointestinal carcinoma. It was also tested in bladder cancer patients (14). The recurrence rate was higher in patients with raised levels of CA-50 antigen than those with normal levels of this antigen. The CA-50 antigen expression also correlated inversely with tumor stage, papillary status, grade of differentiation, and tumor progression (15). DNA ploidy and CA-50 antigen expression tested in combination strongly predicted patient survival. In multivariate analysis using Cox's proportional regression hazards model, CA-50 antigen expression showed an independent prognostic value in pTa andpTl SET.

- MCA

It was established that the higher the MCA positivity and the lower the CA-50 positivity, the worse the prognosis was in bladder cancer patients (16). The use of these tumor markers in combination predict survival better than their use separately.

- **Transferrin receptor**

It is not possible to demonstrate the transferrin receptors (TFR) in normal bladder mucosa, except in the proliferating cells of the basal layer. TFR activity in malignant tissue, however, correlates well with the histological grade and the pathological stage of the tumor (17).

- **CathepsinB**

There is some evidence that cathepsin B (CB), a lysosomal endoproteinase, is associated with tumor invasion. CB immunoreactivity in neoplastic cells revealed a strong correlation with both grade and invasion beyond the lamina propria (18).

Most low-grade papillary tumors display a granular cytoplasmic staining pattern in contrast to high-grade tumors, in which diffuse staining is present in the cytoplasm. Strong tumor cell CB staining is more frequent among recurrent SBT than in patients who remain disease-free. The levels and/or distribution of CB may be of potential value in defining clinically aggressive tumor subsets.

- **Epithelial membrane antigen**

The epithelial membrane antigen immunostaining correlated significantly with patient survival and might provide an additional prognostic information (19).

- **Laminin and type IV collagen**

Laminin, type IV collagen, heparan sulphate proteoglycan (perlecan), and nidogen are the main molecular components of the basal lamina (BL), including that of the urothelial cells. Homotypic and heterotypic interactions between these macromolecules ensure the BL integrity. During tumor cell growth and dissemination, these relationships become modulated.

Invasion of a carcinoma involves the degradation and penetration of the epithelial BL. This phenomenon might be used for histopathologic evaluation of bladder neoplasms. The 5-year survival rate of patients having tumors with an interrupted or absent BL was significantly lower and the rate of progression was greater than that of patients with an intact BL, as revealed by immunohistochemical staining of laminin and type IV collagen (20).

Given the current concept that BL is involved in tumor progression and invasion, it appears reasonable to be tested whether other BL molecules, such as perlecan (21) and nidogen, as well as some BL-bound growth factors, such as basic fibroblast growth factor, may serve as prognostic markers in SBT.

- **E-cadherin**

E-cadherin, an adhesion molecule of homotypic cell junctions, has been shown to behave like an invasion suppressor gene *in vitro*. This may explain the inverse relation between expression of E-cadherin and tumor grade that has been found in certain cancers. In normal urothelium, E-cadherin is expressed homogeneously with a typical membranous staining at cell-cell borders. Decreased E-cadherin expression in bladder tumors correlates with both increased grade and stage and with shorter survival (22). E-cadherin expression may serve as a helpful marker of the clinical aggressiveness of bladder tumors, but it must be tested in large prospective studies to assess its precise clinical relevance.

- **Autocrine motility factor receptor**

Otto *et al* (23) also confirmed, that the down-regulation of E-cadherin might identify the high-risk patients in relation to tumor progression and death of bladder cancer. Parallel to E-cadherin, these authors studied the autocrine motility factor receptor (gp78) expression and found that the negative expression was associated with a low risk of clinical progression in the SET patient group. The dual use of these two antigens may improve early diagnosis of high-risk bladder cancer patients and influence treatment decisions.

- **HLA-DR antigen**

The expression of HLA-DR antigen in bladder cancer cells correlates with tumor grade, stage, lymphatic and venous invasion and may be regarded as an independent prognostic factor of tumor growth and progression (24). The 10-year survival of bladder cancer patients with "many" DR-CC (more than 100 cells on high power field) was significantly higher than those patients, whose tumors were negative for DR-CC (25).

BLOOD GROUP ANTIGENS

- **ABH blood group antigens**

ABH blood group antigens (BGAg) are carbohydrate that may be detected on the surface of erythrocytes and some other human cells, including urothelial cells. Bladder tumor cells lose these antigens in the process of malignant dedifferentiation, and this deletion correlates with the stage, grade and tumor progression (1,26,27). Invasive tumors and carcinoma *in situ* show ABH BGAg deletion which is also demonstrated in SET that progress to a higher stage (28). ABH BGAg expression provides more valuable prognostic information than the tumor grade in relation to recurrences. Both the mean disease-free interval and the recurrence rate in BGAg-negative tumors are significantly higher than in BGAg-positive tumors (29-31). The 10-year survival rate of patients with BGAg-positive tumors is 74.3 %, whereas that of BGAg-negative tumors is 46.5%, the difference being statistically significant. Multivariate analysis of large numbers of patients confirms that ABH BGAg deletion is the most significant prognostic factor in relation to tumor progression, and is not influence by the rest of the clinicopathologic factors (28,29,32-35).

- **Lewis antigens**

The close biosynthetic and structural similarities between the ABH and Lewis BGAg made some authors search for similar relationships between the expression of Lewis substances and the progression of bladder cancer, as was found in ABH BGAg. Accordingly, it was shown that 94 % of normal mucosa specimens and 73 % of SET gave positive reactions with both anti-Le^a and anti-Le^b (36). Abnormal patterns of Lewis reactivity < were observed in 43 % of G3 and in 14 % of Gland G2 bladder tumors. Although there was no direct correlation between ABH and Lewis antigen expression, all bladder tumors which had abnormally low reactivity for these antigens were of high-grade and invasive, the expression of BGAg A and Le^a only having a predictive value in cases of SET (37). However, this test could not predict independently the poor disease outcome.

Thomsen-Friedenreich antigen

The expression of ABH BGAg precursor, Thomsen-Frieden reich antigen (T-antigen), may be abnormal or absent in a variety of bladder tumors. This is a more common finding in non-invasive, low-grade tumors having worse prognosis in relation to recurrences than in non-invasive, low-grade tumors with a low risk for recurrences (38). The abnormal expression of T-antigen correlates significantly with the tumor progression and might serve as an independent prognostic factor (35). T-antigen expression may also be used as a predictive test for evaluating the response of bladder tumors to treatment with BCG and interleukin-2 (39). The overall response rate to BCG and interleukin-2 treatment in patients with T-antigen-positive and T-antigen-negative tumors is found to be 100 % and 33 %, respectively.

GROWTH FACTORS

- **Epidermal growth factor receptors**

The epidermal growth factor (EGF) is detected in high concentrations in the urine, and its receptor, EGFR, may be revealed in urothelial bladder cancer. Preliminary data have indicated that assessment of EGFR status is a method of further subclassifying bladder cancer in relation to disease outcome. A strong positive reaction to EGFR is found almost in a half of the examined bladder tumors. Multivariate analysis has shown that such cases have a significantly worse prognosis in relation to recurrences, progression and tumor-related death (12,40,41). Besides, EGFR was confirmed to be an independent predictor of survival and stage progression (42). Most probably EGFR overexpression is not an early, but a late event in the process of malignant evolution and is usually associated with genetic instability. Further studies are necessary in order to prove convincingly that EGFR analysis gives a more precise prognostic information than the established prognosticators.

ENZYME ACTIVITY

- The activities of phosphofractokinase (PFK), oc-glycerolphosphate dehydrogenase (a-GPDH), and phosphohexose

isomerase (PHI) in tissue samples of bladder tumors showed significant decreases with increased stage and grade (43). Decreased activity of the enzymes PFK, PHI and lactate dehydrogenase was associated with significantly higher risk of tumor progression (44). In effect, it was suggested that the measurements of en2yme activity in SET might help selecting individual patients with a high risk of progression, for adjuvant intravesical treatment.

BIOCHEMICAL PARAMETERS

• The role of carcinoembryonic antigen (CE A) has been reviewed by Ackermann (45) who reported a wide range of CEA levels in the blood of patients suffering from bladder cancer. This test did not find any wide-spread clinical application, because of its low sensitivity and low specificity in cases of urothelial bladder cancer. Increased levels of serum carbohydrate antigen 19.9 might be important in monitoring the natural history of bladder cancer; however, its value as a prognostic indicator is also still questionable (46). A promising tumor marker is the tissue polypeptide antigen (TPA). Except for tumor diagnosis, plasma TPA concentration has showed a good correlation with tumor progression as well as regression after treatment in urothelial bladder cancer (47). Carbin *et al* (48) later suggested the important role of urinary TPA as an indicator of bladder cancer recurrence.

OHCOGEMES AMD TUMOR SUPPRESSOR GENES

• It is well accepted that defects at a number of transcriptional and posttranscriptional steps in a gene product may be involved in the appearance of malignancy or a metastatic phenotype. These include transcript initiation, transcriptional proof-reading, pre-mRNA splicing, and mRNA export from the nucleus to the cytoplasm. Examples of oncogenes and tumor suppressor genes used as prognostic markers in bladder tumors are presented below.

• *c-erbT$-2* oncogene

The *c-erbB-2* gene product is a transmembrane receptor protein with partial homology to EGFR. Amplification or overexpression of the *c-erbB-2* gene have been reported to correlate with poor prognosis in human breast, gastric, and ovarian cancer. Recently, the *c-erbB-2* protein was found to be expressed frequently in the urinary bladder carcinoma as well. A few studies have confirmed that the expression rate of *c-erbB-2* protein corresponded to the advancement of tumor grade and stage and correlated with patient survival (49-51). Tumors without *c-erbB-2* expression had a higher risk of recurrence, and that was independent of the stage and grade of bladder cancer. The 5-year disease-free survival rate is 48.5% for patients with *c-erbB-2* -negative tumors vs 9.7 % for those with *c-erbB-2* -positive tumors (50). On the contrary, the immunohistochemical demonstration of *c-erbB-2* protein overexpression in paraffin-embedded archival material had no prognostic value over already established predictors in transitional cell bladder cancer (52). Therefore, a better understanding of the regulatory mechanisms and physiological properties of *c-erbB-2* protein in the urothelium is required and its precise predictive role in bladder cancer cases should be further clarified.

• p53 gene

The tumor suppressor p53 gene is located in chromosome 17pl3-l and encodes for a 53 kD nuclear protein (p53). The role of p53 is to prevent damage to cells via DNA repair, and to induce apoptotic cell death in case of irreversible damage. Accordingly, alteration of the p53 tumor suppressor gene is the most common genetic abnormality found in human cancer, including bladder cancer. Immunohistochemical detectability of p53 is associated with early events in the malignant evolution, occurring in 48 % of cases of bladder carcinoma *in situ* (53). Other reports show that there are different patterns of staining for p53 protein, according to which tumors may be divided into group A, in which no more than 20 % tumor cells show positive nuclear staining, and group B, with 20 % or more nuclear immunoreactivity. Patients in group B have a higher probability of disease progression and a significantly lower progression-free interval (54-57). Positive staining for p53 is found more frequently in poorly differentiated and in invasive tumors (58,59). Expression of p53 is also closely associated with tumor recurrences. A correlation between mutated p53 and poor survival in the whole group of patients is found as well (60). There are however great variabilities in tumor staining for p53, even with the same antibody (61). The heterogeneity of p5 3-positive cell distribution in the tumors indicates potential for significant sampling errors. The use of immunohistological p53 expression as a discriminating prognostic indicator in pTl transitional cell cancer merits further attention. If the high prognostic value of p53 mutations in SET is confirmed in larger prospective trials, more aggressive therapeutic strategies could be discussed for patients with p53 mutations in their tumor specimens. Correlative studies of expression of p53, a stimulator of apoptosis, and *bcl-2,* a suppressor of apoptosis (see Arumae in this volume of *Biomedical Reviews),* may provide additional prognostic significance in bladder cancer.

• p21 gene

Abnormal activation of *ras* oncogene and the expression of its product p21, a 21 kD protein, are present in a number of human tumors. Using an ABC immunoenzymatic staining technique and a p21 specific monoclonal antibody, Miao *et al* (62)

detected that the morbidity rate due to tumor recurrence in p21-positive patients was only 9 %, whereas in p21-negative patients it was 67 %.

- **MDM2gene**

The human MDM2 gene was recently cloned and located in chromosome 12ql3-14. There is a striking association between MDM2 overexpression and low-stage, low-grade bladder tumors, and with p53 overexpression as well (63). So, simultaneous immunostaining of MDM2 and p53 may be an important diagnostic and prognostic method in patients with bladder cancer.

- **Retinoblastoma gene**

The retinoblastoma tumor suppresor gene (RB1) is located in chromosome 13ql4, and its product is a 110 kD cell cycle-related nuclear protein (pi 10^{Ml}). Interestingly, pi 10^{RB1}, known to be involved information of retinoblastomas, is also present in other tumor tissues, including bladder cancer (64). pi W^{mi} expression was found to be independent of other known prognostic variables (65,66). A low value for the fraction of RB1 protein-positive nuclei is related to a large fraction in S phase, high mitotic index and overexpression of EGFR and mutated p53. In an univariate survival analysis altered expression of RB1 protein and low frequency (< 50 %) of RB1 protein-positive nuclei predicts poor outcome. These results show that RB1 gene, as well asmutation in p53 gene, participate in the growth regulation of human bladder cancer cells *in vivo* and accordingly modify the prognosis.

- **Multidrug resistance gene**

Resistance of malignant cells to cytotoxic agents is often a limiting factor to successful chemotherapy. The classical multidrug resistance (MDR) is characterised by overexpression of a 170 kD transmembrane, transporter glycoprotein named P-glycoprotein (gp!70). The latter acts like a drug extruding pump reducing accumulation of cytotoxic agents inside malignant cells, *rndrl* gene expression is significantly higher in poorly differentiated high-grade tumors, than in well and moderately differentiated, low-grade tumors (67). Still no evidence is found, however, to implicate *mdrl* overexpression as a predictor of tumor recurrence or progression in SET.

- **Nucleolar organisers**

Nucleolar organisers (NOR) are intranucleolar segments of DNA coding for ribosomal RNA. The argyrophilic proteins associated with NOR (AgNOR) allow them to be cytolabelled on paraffin sections. The number of NOR expression (NOR index) is correlated with cellular proliferation and has a diagnostic and prognostic value in neoplastic disease. The NOR index is 4.54 for normal urothelium, 5.89 for non-invasive superficial tumors, 7.33 for invasive superficial rumors, and 9.75 for invasive recurrences (68). These results demonstrate an increase in nucleolar argyrophilia with invasion and indicate the invasive potential of SET which are initially homogeneous for stage and grade. Lipponen *et al* (69) assessed the predictive value of silver stained NOR in 229 patients with transitional cell cancer, followed up for over 10 years. The increased number of NOR in pTa-pTl SET correlated significantly with the recurrence rate and tumor progression and with the patient survival. In a multivariate analysis, AgNOR predict progression independently.

GENETIC ABNORMALITIES

• Some chromosomal abnormalities play a certain role in the development of bladder tumors. Bladder cancer with normal karyotypes remains superficial and continues to exhibit a non-aggressive course, while patients whose tumors have an abnormal karyotype at the time of initial diagnosis, express a much more aggressive course that ultimately develops into invasive disease (70,71).

CD44 GENE TRANSCRIPTS

• Recently, a great advance has been made in understanding alternative splicing, the RNA processing mechanism whereby the primary transcript (pre-mRNA) of a gene is processed by spliceosomes, removing introns, and adjoining specific combinations of exons, toyeild different copies of mRNA, leading to the expression of multiple proteins from a single gene, i.e. alternatively spliced gene products. The most notable example to date for attributing pathological consequences to the expression of altenatively spliced gene products is the role of the cell adhesion molecule CD44 in tumor growth, invasion, and metastasis. The CD44 gene is 50-60 kbp long, resides on chromosome 11, and is composed of at least 20 exons, ten or more of which can be alternatively spliced to produce various isoforms. Matsumura *et al* (72,73) described for the first that exfoliated tumor cells in the urine of bladder cancer patients exibited CD44 transcripts having retained the variant intron 9. The abnormal products of this gene can be detected with the technique of reverse transcription (RT)-polymerase chain reaction (PCR) followed by monoclonal or polyclonal antibodies to peptide sequences encoded by the variant exons of the gene. In effect, intron retention hypothesis (73) could form the basis for a new, specific and effective non-invasive diagnostic and prognostic test for the bladder cancer.

CONCLUSIONS

• It is evident that various biological tumor markers, whose expression correlates with the recurrence and progression rates

and patient survival, may be used as objective prognosticates of the malignant evolution of SET. Unfortunately, a complete consensus concerning the clinical application of most of the already established tumor markers does not exist yet. The controversy available in the published results of clinical trials is most probably due to the limited number of the patients, incorrect patient selection, lack of standardized histochemical techniques, etc. That is why most of the aforementioned biological tumor markers still are at a preclinical phase of application and cannot be used alone as basic criteria when the treatment schedules for initial therapy and metaphylaxis have to be determined. Coordinated studies using selected monoclonal immunostaining, RT-PCR, *in situ* hybridization, and microwave antigen retrieval methods (74) may further be organized, searching for state-of-the-art tumor markers in bladder cancer. Together with the rapid development of medical genetics, leading to a more profound knowledge of the human genome and of the molecular aspects of cancerogenesis, that will allow an widespread clinical use of biological tumor markers in close future. In such a way, the final goal of the prognostic assessment - an improvement of the individual counselling, may be achieved. Associated with the clinical application of new, more efficient, local chemotherapeutics and immunomodulators and optimal therapeutic schedules, that will lead to a significant reduction of the recurrences and tumor progression in cases of bladder cancer. Biological tumor markers seem to be therefore some of the most perspective arms in the eternal fight against this insidious malignancy.

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