Expression of the Endothelin-axis in the different histologic subtypes of renal cell carcinoma: A tissue microarray analysis

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Abstract. Endothelin-1 and its receptors ET_AR and ET_BR, commonly referred to as the Endothelin-axis, are emerging to play a role in cancer. The Endothelin-axis has been shown to be involved in proliferation, angiogenesis and metastasis in various human tumours. To assess the role of the Endothelin-axis in renal cell carcinoma, we analysed its expression in archival tumour tissue of 183 patients. Representative tumour blocks were selected for constructing a tissue microarray. Paraffin sections were assessed immunohistochemically using monoclonal and polyclonal antibodies for Endothelin-1, ET_AR and ET_BR. Staining intensities were analysed semi-quantitatively and the results were correlated with various histopathologic factors. Overexpression of Endothelin-1, ET_AR and ET_BR was identified in 12.8%, 84.1% and 93.3% of cases, respectively. No association with pathological tumour stage and histologic grading was found. Papillary renal cell carcinomas expressed highly significantly more Endothelin-1 than clear cell renal cell carcinomas (34.5% vs. 6.7%, $p<0.001$), while there was no difference between ET_AR- and ET_BR-expression in these histologic subtypes. However, ET_AR tended to be overexpressed in the subgroup of G3-tumours ($p=0.044$). Studies are underway assessing the role of the Endothelin-axis and its potential use as a molecular target in renal cell carcinoma.

Introduction

In the last 50 years, the number of people diagnosed with renal cell carcinoma (RCC) has dramatically increased: it is estimated that the prevalence in the US has risen by 126% in this period. The American Cancer Society reported about 31,900 new cases of cancer involving the kidney in 2003, and 35,710 in 2004. (1). The current classification system for RCC includes a number of histologic subtypes (2). The two most common ones are clear cell renal cell carcinoma (ccRCC), representing approximately 70-80% of patients, and papillary renal cell carcinoma (pRCC), which is observed in only 10-15% of patients (2,3). Currently, radical nephrectomy is the therapeutic gold standard. However, the prognosis remains unfavourable, especially for metastatic disease (1). Therefore, molecular markers need to be determined to allow better discrimination of aggressive tumour phenotypes and to identify candidates for a targeted therapeutic approach in the future.

Among targets that have emerged to play an important role in tumour biology are the endothelins and their receptors. They consist of three small and multifunctional vasoconstrictor (21-amino acid) peptides: ET-1, ET-2 and ET-3. ET-1 is produced by a variety of normal cells, including endothelial cells, vascular smooth muscle cells and various epithelial tissues, while ET-2 is expressed mainly in intestine and kidney, and ET-3 in the brain (4). All of them exert their physiologic effect via two high-affinity, G-protein-coupled receptors, ET_AR and ET_BR. The combination of ET-1, the best characterised endothelin to date and the two receptors is referred to as the (Endothelin-) ET-axis. Kusuhara *et al* were the first to demonstrate ET-1 production in tumour cell lines (5), and since then a growing role for ET-1 in tumour biology has been postulated for several tumour entities (6,7). By different mechanisms, ET receptor activation promotes tumour associated functions through ET_BR such as cell proliferation and migration, whereas stimulation of vascular smooth muscle, pericyte mitogenesis, and production of vascular endothelial growth factor (VEGF), which leads to endothelial cell proliferation and vascular permeability by increasing the levels of hypoxia-inducible factor (HIF)-1α, are predominantly accomplished by ET_AR (8-10).

Nelson *et al* were the first to show that ET-1 is a potentially important factor in advanced prostate cancer progression (11), which later led to initiating a randomized, placebo-controlled phase-II trial with Atrasentan (ABT-627), a selective ET_AR antagonist. In an intent-to-treat analysis of results, a trend towards prolongation of disease progression and a statistically significant delay in PSA progression were demonstrated (12). Recently, preliminary results were reported from phase-III

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trials with Atrasentan. They revealed a trend toward delayed time to progression and significantly delayed elevation of bone turnover markers (13).

In invasive bladder cancer, our group was able to report a high expression of the ET-axis at the mRNA and protein level (9). In a concomitant study we showed that both ET receptors were overexpressed, ET_BR predominating. Expression of ET_BR was associated with several parameters of favourable tumour types and a better prognosis as well as a longer disease-free survival. To assess the role of ET_AR as a molecular target in bladder cancer, we carried out a preclinical study on bladder tumour xenografts with Atrasentan. Compared to the placebo group, a diminished growth rate with an increased percentage of necrosis in the tumour tissue was reported, although no significant cytoreduction was noted. Studies at our department are ongoing to evaluate the role of Atrasentan to serve future targeted molecular therapy. Results are expected in the near future.

To date, to the best of our knowledge, there have been published only three small reports about the expression of the ET-axis in RCC (14-16). Although the results showed activity for the ET-axis, they were not sufficient to define its role in RCC and the different histologic subtypes accurately and to evaluate ET-1 and its receptors to serve as potential molecular targets for further clinical trials. We therefore undertook this study to analyse the expression of the ET-axis and its impact on pathologic parameters in a large group of patients undergoing nephrectomy.

Materials and methods

Patients and tumour specimens. Patients (n=183) undergoing radical nephrectomy for RCC at our institution were included in the study. Men (n=133) and women (n=50) were operated on between 1991 and 2004; median age at surgery was 61 (range, 25-95) years. Histologic slides and formalin-fixed, paraffin-embedded tumour tissue blocks from all 183 patients were obtained from the files at the Institute of Pathology (University of Münster). All slides were reviewed, and representative tumour tissue blocks were selected for immunohistochemistry. A database comprising histopathologic data regarding tumour stage, histologic grading and histology type was created (Table I).

For each of the 183 cases we selected a representative tumour block as a donor block for the tissue microarray (TMA). Using an H&E-stained slide, two morphologically representative regions were defined for each of the 183 tumour samples. From these regions, cylindrical core tissue specimens (diameter =0.6 mm) were obtained and arrayed precisely into a new recipient paraffin block (20x35 mm) using a custom-built precision instrument (Beecher Instruments, Silver Spring, MD). From the 366 tumour samples available, four tissue array blocks were prepared.

Immunohistochemistry for ET-1, ET_AR and ET_BR. The paraffin-embedded tumour tissue blocks were cut into 3-μm slices and mounted on poly-L-lysine-coated glass slides. Tissue slides were dewaxed in xylene, rehydrated in a graded series of alcohol and rinsed in 0.01 M Tris buffer (pH 7.3). Immunohistochemical staining for ET-1, ET_AR and ET_BR was performed in a multistep semiautomatic procedure (Ventana NexES automated immunohistochemistry system) as described previously by Wulfing *et al* (17). Briefly, two polyclonal antibodies for ET_AR and ET_BR (Alexis Biochemicals Corporation, Lausen, Switzerland) at a dilution of 1:100 for 30 min were used. For ET-1, a monoclonal mouse antibody (CloneTR.ET.48.5, Affinity Bioreagents, Golden, USA) was applied at a 1:500 dilution (25 min) after pretreatment with a steamer for antigen retrieval (Multi-Gourmet, Braun, no. 3216). Specimens were then incubated with a rabbit anti-sheep secondary antibody (Dako; 1:500 for 25 min), followed by blocking of endogenous peroxidase (H_2O_2 for 10 min) and incubation with an Envison™ Detection kit (25 min). The enzyme reaction was developed with Chromogen-DAB (2x5 min). Finally, the specimens were counterstained with hematoxylin and mounted with Kayser's glycerine. Ovarian cancer tissue served as positive control for ET-1 staining, prostate cancer tissue for ET_AR, and smooth muscle tissue for ET_BR. The specificity of the antibodies was confirmed using omission of the primary antibodies and replacement of the primary antibodies by IgG of the respective species as negative controls. Immunohistochemical staining was independently scored by two investigators from 366 array cores (E.E., H.B.). According to the literature (18), intensities of ET-1, ET_AR and ET_BR were classified semiquantitatively into different grades on an arbitrary four-tiered scale of 0 to 3+. We defined tumour samples with weak (1+), moderate (2+) or strong (3+) immunostaining intensity to have an elevated ET-1, ET_AR or ET_BR expression, and thus to be positive (Fig. 1).

Data analysis. Staining intensity was evaluated semiquantitatively in a blind fashion. For statistical analysis SPSS for Windows™ (Version 13.0) was used. All histopathologic parameters were correlated with staining results by means of cross-tables applying Chi-Square and Fisher's exact test. Correlations between the two different samples from identical tumours were tested to investigate variance of expression. A $p<0.05$ was considered statistically significant.

Table I. Distribution of tumor size and histologic grade in the reported series of RCC specimens (n=183).

Pathological parameters [n (%)]	
Tumor stage[a]	
pT1	76 (42.7%)
pT2	21 (11.8%)
pT3	80 (44.9%)
pT4	1 (0.6%)
Histologic grade[b]	
G1	20 (12.0%)
G2	111 (66.5%)
G3	36 (21.6%)

[a]Information on pT stage was available in 178 of 183 (97.3%) patients. [b]Information on grading was available in 167 of 183 (91.3%) patients.

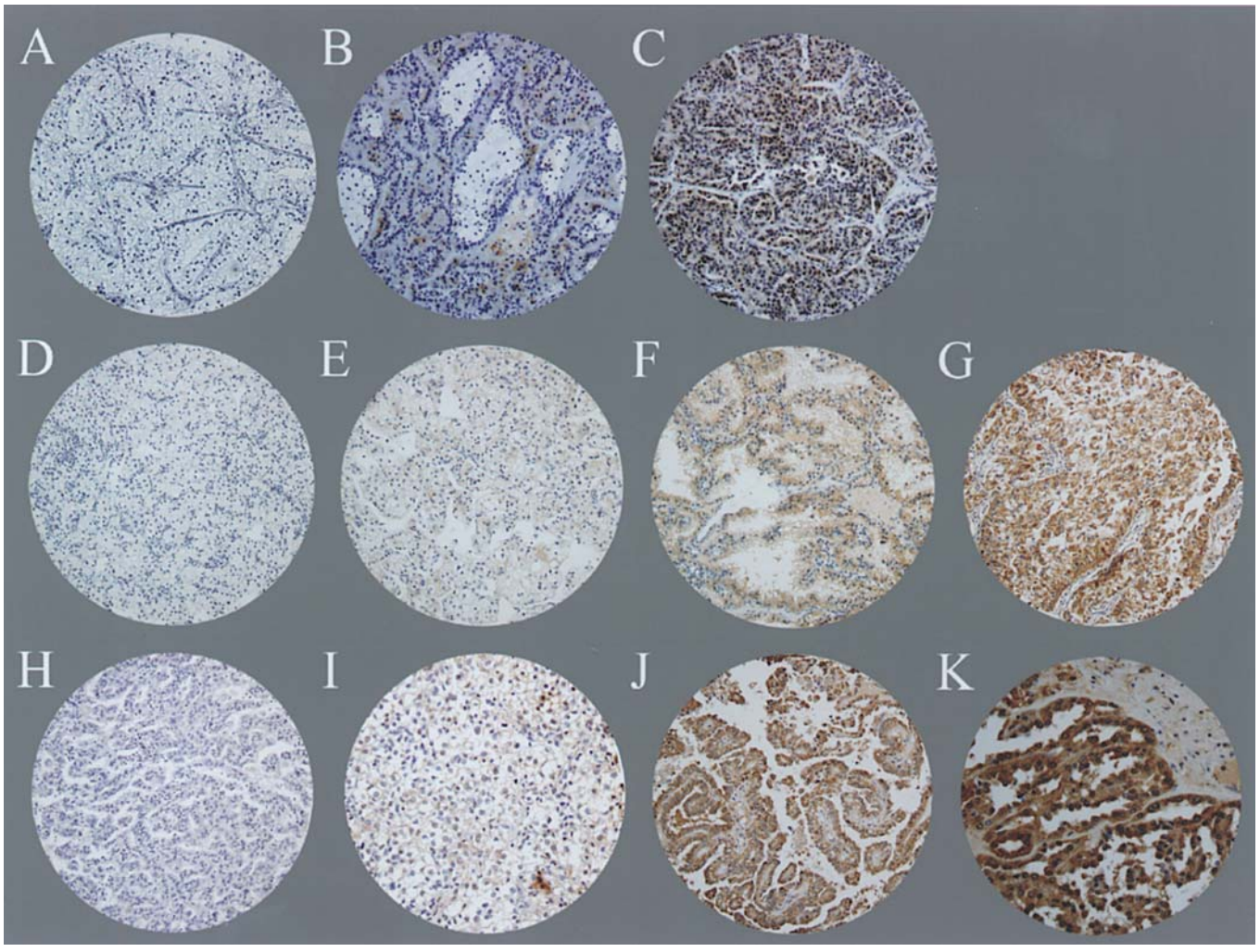

Figure 1. Representative examples of staining for ET-1 (A, negative; B, weak; C, moderate), ET_AR (D, negative; E, weak; F, moderate; G, strong) and ET_BR (H, negative; I, weak; J, moderate; K, strong) (images reduced from x20).

Results

Immunohistochemical analysis of ET-1, ET_AR and ET_BR expression. From the 183 patients, ET-1, ET_AR and ET_BR staining status was available in 180 (98.4%), 182 (99.5%) and 180 (98.4%) cases, respectively. In the missing cases, tissue had been lost during steamer-pre-treatment or the core samples did not contain a sufficient number of tumour cells. Staining intensity of ET-1, ET_AR, and ET_BR among different samples varied from complete absence of staining to strong staining. Endothelin-1 staining was identified in 12.8% of cases; expression of ET_AR and ET_BR was increasingly frequent (84.1% and 93.3%, respectively). Predominantly the staining patterns were either weak or moderate (Table II).

Table II. Distribution of staining patterns for ET-1, ET_AR and ET_BR.

	n (%)		
	ET-1 (n=180)	ET_AR (n=182)	ET_BR (n=183)
Score			
0	157 (87.2%)	29 (15.9%)	12 (6.7%)
1+	21 (11.7%)	105 (57.8%)	77 (42.7%)
2+	2 (1.1%)	47 (25.8%)	84 (46.7%)
3+	0 (0.0%)	1 (0.5%)	7 (3.9%)
Negative (score 0)	157 (87.2%)	29 (15.9%)	12 (6,7%)
Positive (score 1-3)	23 (12.8%)	153 (84.1%)	171 (93,3%)

Correlation with pathologic parameters. From the 183 specimens, we were able to define the histologic subtype in 176 (96.2%) of cases. Of the RCCs, 135 (73.8%) were of clear cell and 30 (17.1%) were of papillary origin. Sarcomatoid tumours were found in 6 (3.4%) and chromophobe tumours in 5 (2.8%) cases. ET-1 expression did not correlate with tumour stage and histologic grade; pRCC expressed highly significantly more ET-1 than ccRCC (34.5% vs. 6.7%, $p<0.001$). Although there was no association with tumour size or histologic subtype, ET_AR tended to be overexpressed in the subgroup of G3-tumors ($p=0.044$). ET_BR was the

Table III. Association of positive ET-1, ET_AR and ET_BR expression with histopathological variables.

Pathological variables	ET-1 staining n positive/total (%)	p[a]	ET_AR staining n positive/total (%)	p[a]	ET_BR staining n positive/total (%)	p[a]
Tumor stage						
pT1	10/76 (13.2%)	0.65	63/76 (82.9%)	0.69	73/76 (96.1%)	0.43
pT2	4/21 (19.0%)		16/21 (76.2%)		18/21 (85.7%)	
pT3	8/77 (10.4%)		69/79 (87.3%)		72/77 (93.5%)	
pT4	0/1 (0.0%)		1/1 (100.0%)		1/1 (100.0%)	
Histologic grade						
G1	5/20 (25.0%)	0.31	17/20 (85,0%)	0.04	19/20 (95%)	0.57
G2	12/109 (11.0%)		90/119 (81,8%)		102/109 (93.6%)	
G3	4/35 (11.4%)		34/36 (94.4%)		32/35 (91.4%)	
Histology type						
ccRCC	9/133 (6.8%)	0.001	112/135 (83.0%)	0.52	126/133 (94.7%)	0.13
pRCC	10/29 (34.5%)		26/29 (89.7%)		27/29 (93.1%)	
Sarcomatoid	2/6 (33.3%)		6/6 (100.0%)		5/6 (83.3%)	
Chromophobe	0/5 (0.0%)		3/5 (60.0%)		1/5 (20.0%)	

[a]Fisher's exact test.

predominant receptor in this TMA but it still did not show any association with tumour size, histologic grading or histologic subtype. A comprehensive summary of the staining patterns of ET-1, ET_AR and ET_BR and their association with several pathologic variables is shown in Table III.

Discussion

RCC is the sixth leading cause of cancer deaths and the most lethal urologic cancer in the developed nations. RCC accounts for 2-3% of cancer incidence and results in over 100,000 worldwide deaths annually (19). RCC age-adjusted incidence has been rising for the past 30 years within the US and most European nations at an annual rate of approximately 3% (19). For those who present with metastases, the overall clinical course of RCC varies; approximately 50% of patients survive less than 1 year and 10% survive for over 5 years (20). In the past two decades, management of the disease has undergone considerable change. Chemotherapy has consistently been an ineffective form of treatment, and until recently, the only effective treatment for metastatic disease was cytokine-based immunotherapy with interferon (IFN)-α or interleukin (IL)-2, which have a response rate of approximately 15% (21). Advances in understanding tumour biology and genetics of metastatic RCC have led to several novel targeted approaches with higher response rates for agents such as Sunitinib (SU11248) and Sorafenib (BAY 43-9006), which were recently approved in the US and some European states (22). In phase II clinical trials these targeted agents had an overall response rate of up to 40% (22). Unfortunately, the clinical response to these agents was not permanent; rather the time to progression was, on average, approximately 6-12 months (22). The results of the clinical trials led to the statement that further molecular targets need to be identified for targeted therapy and improve the prognosis of patients with metastatic RCC.

ET-1 and its receptors ET_AR and ET_BR, have recently been demonstrated to be overexpressed in various human tumours such as bladder, ovarian, cervical, breast, colon and lung cancers, as well as in melanomas (6,7). It has been clearly shown that the ET-axis functions in the growth and progression of cancer by influencing apoptosis, angiogenesis and several growth factors (6). The emerging role of the ET-axis in cancer has led to the development of selective ET receptor antagonists for targeted molecular therapy, and results of clinical trials were promising for Atrasentan in the treatment of hormone-refractory prostate cancer (13).

In the kidney, ET-1 is secreted by a number of different tissue compartments, including blood vessels, glomeruli, tubules and collecting ducts. ET_AR and ET_BR are found in the same cells, but also exist in juxtaglomerular and mesangial cells (23). The most common histologic subtypes of RCC are ccRCC and pRCC. A number of distinguishing features at the macroscopic, microscopic and cytogenetic levels have been recognized that suggest these tumours differ significantly. They include the hypervascular nature of ccRCC compared with the characteristically hypovascular appearance of pRCC, the presence of infiltrating lymphocytes and macrophages in pRCC but not ccRCC, and the increased incidence of necrosis in patients with pRCC but not ccRCC (14). Distinct karyotopic aberrations also set these subtypes apart, such as loss of 3p in ccRCC compared with trisomies of 7, 12, 16, 17 and 20 and loss of the Y chromosome in pRCC (2,3,24). Some authors have reported less favourable clinical outcomes in patients with

papillary tumours (25). Other authors suggested a significantly favourable outcome for patients with pRCC compared to ccRCC (26). In the latest study to date, Patard *et al* (27) initiated a multicenter study with 4063 patients to examine the prognostic value of histologic subtypes in RCC; papillary and clear cell tumours were not found to have a significantly different outcome in distant disease.

In RCC, to our knowledge, the ET-axis is described in only three small studies. In 1999 Thevarajah *et al* examined its expression in six RCC cell lines by enzyme linked immunosorbent assay (ELISA) for ET-1 and RT-PCR confirmed by Southern blot hybridization for ET_AR and ET_BR mRNA. Relatively large amounts of ET-1 were secreted. All cell lines expressed ET_AR mRNA, while three cell lines also expressed ET_BR mRNA. ET-1 binding was limited to ET_AR (15). In another small study with 35 specimens, Douglas *et al* demonstrated gene expression for preproendothelin (PPET)-1, -2 and -3, the receptors ET_AR, and ET_BR and endothelin-converting-enzyme (ECE)-1 and -2. PPET-1 was upregulated in ccRCC and downregulated in pRCC. ET_AR was downregulated significantly in pRCC (14). Recently, Pflug *et al* were able to find relatively high levels of ET-1 and ET_AR mRNA in six RCC cell lines, while 3/6 cell lines also expressed ET_BR mRNA. Furthermore they demonstrated that ET-1 signaling in RCC induces protection from apoptosis through inducing the P13-kinase pathway and activating Akt. Finally, they concluded that the use of ET_AR antagonists to inhibit the antiapoptotic functions of ET-1 signaling may induce increased sensitivity to chemotherapeutics *in vivo* and recommended therapeutic targeting of ET_AR as a novel treatment strategy for RCC (16).

In the present study, which is the first to apply standard immunohistochemical techniques, ET-1 was highly significantly overexpressed in pRCC compared to ccRCC ($p<0.001$). These data are controversial to former findings describing gene expression (14), where PPET-1 was overexpressed in ccRCC and impaired in pRCC. Furthermore Douglas *et al* described a downregulation of ET_AR in pRCC, which again cannot be confirmed by our results (14). Reviewing recent data, ET-1 is synthesised via proteolytic cleavage of PPET-1, which is facilitated by the metalloproteinase, endothelin converting enzyme (ECE). Entering this pathway, ET-1 modulates mitogenesis, apoptosis, angiogenesis, tumour invasion and development of metastasis with high affinity to its receptor ET_AR, while ET_BR does not show selective affinity for any of the ET subtypes (ET-1, ET-2 and ET-3) (28). ET-1 has been shown to stimulate the growth of several human cancer cell lines *in vitro* (29-31) via ET_AR in epithelial tumours (29,30) and via ET_BR in melanoma cells (31). *In vivo*, antagonism of ET_BR was shown to significantly slow down melanoma tumour growth in nude mice (32). ET-1 also acts as a mitogen for both endothelial cells and vascular smooth muscle cells and stimulates VEGF production by other cell types (18). Bek *et al* were able to inhibit ET-1 stimulated angiogenesis by ET_AR antagonism in mice (33). Overexpression of ET-1 is associated with tumour invasion and metastases; it modulates the growth of bone metastases from prostate cancer (34). From the evidence to date on ET-1 expression in epithelial tumours, it appears that selective ET_AR antagonism provides the most likely effective method of endothelin inhibition (28). As renal cell carcinoma belongs to the group of epithelial tumours and ET_AR is overexpressed in 84.1% of cases, the effects in tumour biology described above might be blocked by ET_AR antagonism in this tumour entity. Patients with pRCC and significant overexpression of ET-1 (34.5%) compared to ccRCC (6.7%) ($p<0.001$) or patients with G3-tumors of either histologic subtype and upregulated ET_AR expression (94.4%) ($p=0.044$) as in the present study might benefit from this type of molecular targeted therapy. The other receptor, ET_BR, is predominantly expressed in 93.3% of cases; this is consistent with data on breast cancer, where ET_BR was expressed more frequently than ET_AR (53.4% vs. 46.5%, repectively) (17). In accordance with our data, ET_BR is also the predominant receptor in melanoma and lung cancer (6,7).

Our results show that the ET-axis is overexpressed in RCC with ET-1 being highly significantly upregulated in pRCC and ET_AR in the subgroup of G3-tumors. The exact biochemical link between expression of the ET-axis, distribution and regulation of the ET receptors, and influence on apoptosis, angiogenesis and proliferation in RCC remains unclear. The effect of the ET-axis on clinical outcome and prognosis is currently under investigation at our institution. Moreover, further preclinical and clinical studies will be initiated to elucidate whether the ET-axis can play a role as a prognostic marker and molecular target for renal cell carcinoma.

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