Expression of the cell-cycle regulatory proteins (cyclins D1 and E) in endometrial carcinomas: correlations with hormone receptor status, proliferating indices, tumor suppressor gene products (p53, pRb), and clinicopathological parameters

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Summary

Purpose of investigation: This study aimed to investigate the immunohistochemical expression of cyclins D1 and E in normal, hyperplastic and neoplastic endometrium, and their correlation with proliferative activity and clinicopathological features.

Methods: We carried out immunohistochemical techniques on archived material of formalin-fixed paraffin-embedded tissues using the antibodies against the cyclins D1 and E, PR-ER, p53, Ki67 (MIB1) and pRb with the streptavidin-biotin-peroxidase method in a total of 20 cases of normal endometrium, 32 cases of hyperplastic endometrium and 66 cases of endometrial carcinomas.

Results: Cyclin D1 and E immunoreactivity was observed in the nuclei of tumour cells in 18.2% and 39.1%, respectively, of the cases of endometrial carcinomas. Cyclin D1 labelling index was not significantly correlated with any of the clinicopathologic parameters examined. However, there was a significant correlation between the cyclin E labelling index and histological grade of carcinoma ($p = 0.00096$), which increased significantly with histological grades of malignancy. We also detected a significant correlation between cyclin E and PCNA ($p < 0.0001$) as well as with the tumor suppressor genes p53 and pRb ($p = 0.052$ and 0.0002, respectively) in endometrioid endometrial carcinoma.

Conclusion: Our results indicate that cyclin E overexpression may be involved in the development and/or proliferation and differentiation of human endometrioid endometrial carcinoma. Immunoexpression of cyclin D1 does not appear to be associated with cell-cycle progression in the benign or malignant endometrium.

Key words: Cyclin D1; Cyclin E; p53, pRb; Endometrial carcinoma.

Introduction

Endometrial carcinoma represents the most common invasive gynaecologic malignancy. In the USA it was estimated to account for 38,300 cases and 6,600 deaths in 2001 [1]. Surgical stage, histologic grade of malignancy and subtype, myometrial penetration, nodal involvement, and peritoneal cytology may be relative reliable prognostic factors. Current data support the use of cellular markers, hormone receptor status, proliferative activity, ploidy and molecular markers (c-erbB-2 and p53) as a complement to traditional surgical and pathologic variables for identifying high-risk patients with cancer [2]. The normal human endometrium is characterized by phases of active proliferation and differentiation, which are controlled by ovarian steroid hormones. These phases are also characterized by oscillating levels of cell-cycle regulating proteins [3]. The cell cycle and aberrations have been the subject of intense research in recent years. p53 remains the most widely known mutated cell cycle regulator in human malignancies, but the cyclins are important in the transformation and progression of many types of malignancies [4]. The cyclins ensure normal cell cycling by combining with cyclin-dependent kinases to form heterodimeric molecules, which allow an orderly progression through the different phases of the cell cycle. The division of eukaryotic cells is regulated by a family of protein kinases known as cyclin-dependent kinases (CDKs) [5]. The sequential activation of individual members of this family and their consequent phosphorylation of critical substrates promotes orderly progression through the cell cycle [5, 6]. The deregulation of cell proliferation represents a hallmark of neoplastic transformation. Alteration in growth control pathways must transform into changes in the cell cycle by regulatory machinery, but the mechanism through which this occurs is largely unknown. Human D1 cyclin gene was independently discovered as the gene located at a chromosome rearrangement in a parathyroid tumor, as a candidate bcl-1 oncogene, and as a gene whose transcription is induced in the early G1 phase in response to extracellular mitogens. To carry out its function, cyclin D1 is associated with Cdk4, its catalytic subunit [5, 7]. The kinase activity of the cyclin D1-Cdk4 complex is maximal in the early to mild G1 phase, and it is thought that this kinase phosphorylates and inactivates the retinoblastoma protein (pRb) during G1 [7]. Amplification of cyclin D1 has been reported as the subset of various types of human tumors [4, 6, 8].

Cyclin E, located on chromosome 19q12-13, produces a 395 amino-acid protein that contributes to normal cell proliferation and development, and is required for efficient DNA replication [9]. Cyclin E associates with Cdk2 and activates in Serine/Threonine kinase activity shortly

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before entry into the S phase [9, 10]. Overexpression of cyclin E decreases cell size, diminishes the requirement for growth factors, and accelerates the G1 phase of the cell cycle. In addition, the cyclin E-Cdk2 complex has been shown to be a target for regulators of G1 progression such as p21 and p27 [9].

The retinoblastoma (Rb) gene, which is located on chromosome 13q14, was the first tumor suppressor gene to be isolated. Loss of heterozygosity and loss of expression of the Rb gene are seen during the development of retinoblastoma and several other types of human cancers. This protein is hypophosphorylated during the G1 phase and hyperphosphorylated in the S, G2 and M phases of the cell cycle. It is thought that this phosphorylation blocks an inhibitory function of the Rb protein on progression through the later phases of the cycle [11].

In the present study we investigated the immunohistochemical expression of cyclins D1 and E in normal endometrium (proliferative and secretory), hyperplastic (simple, complex and atypical), and neoplastic epithelium in order to clarify their role in endometrioid endometrial cancer development and progression. In addition, we studied the correlation between cyclins D1 and E with traditional clinicopathological prognostic factors as well as with markers of potential prognostic value, such as hormone receptor status (ER-PE), tumor suppressor genes (p53 and pRb), and proliferation associated indices (ki67 and PCNA).

Materials and Methods

A total of 118 cases were collected from the archives of the Departments of Pathology of the University Hospital and the General Hospital "G. Hatzikosta" of Ioannina. The cases were selected to represent a spectrum of endometrial changes including proliferative (n = 12) and secretory endometrium (n = 8), hyperplasias (n = 32) and endometrial carcinomas (n = 66). All the tissues examined had been fixed in 10% formalin and embedded in paraffin wax. Sections were cut and stained with hematoxylin and eosin (H&E). The diagnoses were reviewed and the carcinomas were graded and staged according to the revised FIGO (International Federation of Gynecology and Obstetric) criteria [12]. Classification of hyperplasia was based on the criteria of the World Health Organization (WHO) [13].

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections. The slides were cut at 4 μm, deparaffinised in xylene, and dehydrated through graded alcohols. For the detection of cylins D1 (clone DCS-6) and E (clone 13A3), pRb, p53 and Ki67 (MIB1) slides were immersed in citrate buffer (10 mM, pH = 6.0) in plastic Coplin jars and subjected to microwave irradiation twice for 15 min. The heat-mediated antigen retrieval method was not used for PCNA and PR-ER staining. Subsequently, all sections were treated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and the method involving the streptavidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine H_2O_2 substrate for 5 min. The slides were counterstained in Harris haematoxylin, dehydrated and mounted. The antibody sources and dilutions are shown in Table 1.

Table 1. — *Antibodies used.*

Antibody	Supplier	Dilution	Incubation time
Cyclin D1	Novocastra	1:10	One hour*
Cyclin E	Novocastra	1:10	One hour*
p53 (IgG2b)	Ylem	1:200	One hour*
pRB 9AB-5)	Oncogene	1:80	Overnight
ER	DAKO	1:50	One hour*
PR	DAKO	1:75	One hour
PCNA (PC10)	DAKO	1:50	One hour
Ki67 (MIB1)	Ylem	1:5	Overnight

*with microwave antigen retrieval.

Immunohistochemical evaluation of cyclin D1 and E

The slides were assessed for reactivity in the nuclei of endometrial epithelial cells and finally the immunoreeactivity was interpreted by light microscopic examination and evaluated by two observers (AM, EI). Differences in interpretation were reconciled by re-review of slides separately or jointly at a double-headed microscope. The staining was evaluated only in areas with well-preserved tissue morphology and away from necrosis and artifacts. Staining was found in the nucleus, and tumor cells showed a range of intensities of staining. Only nuclear staining was evaluated; cytoplasmic reactivity, if present, was disregarded. Every stained nucleus was considered positive, irrespective of intensity. The cut-off for the expression of cyclin D1 and E was < 5%, 5-50% and > 50% of stained cells, which is in about the same range as reported in other studies [14].

ER and PR immunostaining

ER and PR expression was evaluated by examination of the positive epithelial cell nuclei (0, no positive cells; 1, < 10%; 2, 11-50%; 3, 51-80%; and 4, > 80%). In accordance with Segreti *et al.* [15] an immunoreactivity score of 2 and higher was considered to be positive.

p53 immunoreactivity

Based on other studies only cases showing at least 10% immunoreactivity of endometrial cell nuclei were considered to be p53 positive [16].

MIB1 and PCNA immunostaining

Only nuclear epithelial staining was considered positive for MIB1 and PCNA expression and for statistical analysis the cases were divided into groups using a semi-quantitative approach; into two groups for MIB1 (< 10% and > 10% of positive cells), and into three groups for PCNA (< 10%, 10-50% and > 50%) of positive cells) [17].

pRb immunostaining

We considered tissue samples as having a positive pRb phenotype only when pure nuclear staining was demonstrated. Cases were scored as pRb negative if nuclear staining was focal (< 10% of cells). Cases were scored as pRb positive if nuclear staining was heterogeneous (> 10% of positive cells) [18].

Statistical analysis

All data were entered into a microcomputer and statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) for windows (version 6). The association of various variables was confirmed using the Student's t-test, one-way analysis of variance (ANOVA), logistic regression and x^2 test; a p value ≤ 0.05 was considered statistically significant.

Results

Cyclin D1 expression in normal endometrium. In the proliferative phase, nuclear expression of cyclin D1 was observed in 8/12 (12.1%) of the cases. No correlation between its expression with the other examined parameters and biological markers was found. In the secretory phase of the endometrium, positive expression of cyclin D1 was observed in 1/8 (12.5%) of the cases. A statistically significant positive correlation between the expression of cyclin D1, p53 and pRb ($p = 0.046$ and $p = 0.0054$, respectively) was detected in this phase.

Cyclin D1 expression in hyperplastic cases. In hyperplasias the positive expression of cyclin D1 was found in 5/32 (15.6%) of the cases. A statistically significant positive relationship of cyclin D1 expression with p53 was observed ($p = 0.052$).

Cyclin D1 expression in endometrial carcinoma. Absence to low cyclin D1 expression (< 5% positive tumor cells) was observed in 54/66 (81.8%) of the carcinomas; focal to moderate immunoreactivity (5-50% positive tumor cells) 8/66 (12.1%) and strong immunoexpression (> 50%) in 4/66 (6.1%) of the cases (Figure 1). There were no statistically significant differences of cyclin D1 expression between normal, hyperplastic and malignant endometrium. No statistically significant positive relationship of its expression was found with FIGO stage, grade, or hormone receptor status nor with the suppressor genes (p53 and pRb) and proliferating indices (PCNA and MIB1). The data are shown in Tables 2 and 3.

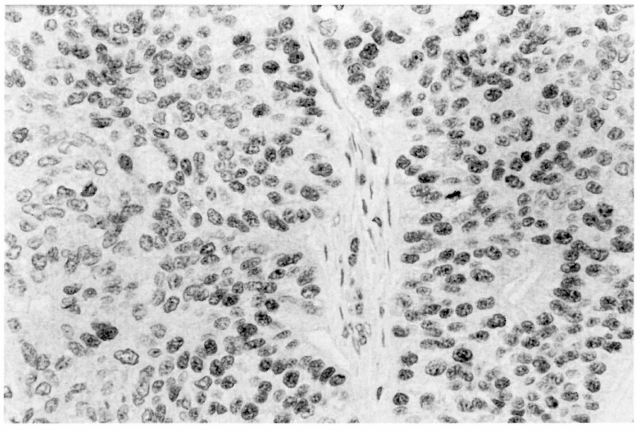
Cyclin E expression in normal endometrium. In the proliferative phase, nuclear expression of cyclin E was observed in 6/12 (50%) of the cases. A positive correlation between cyclin E expression and PCNA was detected ($p = 0.061$) in this phase. No correlation with the other examined biological markers was found. In the secretory phase, positive expression of cyclin E was observed in 5/7 (71.4%) of the cases. A statistically significance positive relationship of cyclin E expression with p53 was observed in this phase ($p < 0.0001$).

Table 2. — *Correlations of cyclin D1 and E expression with clinicopathological features in endometrial carcinomas.*

	Cyclin D1		p value	Cyclin E		p value
	< 5	> 5		< 5	> 5	
Mens. status						
< 45	3	1		3		
45-55	12	3	NS	10	5	NS
> 55	34	8		22	20	
Grade						
1	12	5	NS	11	7	
2	25	3		19	7	
3	14	4		6	11	= 0.00096
FIGO stage						
I	26	5		16	14	
II	11	1	NS	8	4	NS
III	7	2		5	4	
IV	1	–		1		
Vessel Involvement						
–	30	6	NS	22	13	NS
+	17	2		9	10	
Squamous M						
–	26	3	NS	22	6	NS
+	9	2		7	3	

Table 3. — *Cyclin D1 and E expression in correlation with other biological markers in endometrial carcinomas.*

	Cyclin D1		p value	Cyclin E		p value
	< 5	> 5		< 5	> 5	
p53						
< 10	39	19	NS	32	14	= 0.052
> 10	12	3		5	10	
Rb						
< 10	41	7	NS	31	15	= 0.002
> 10	11	5		7	9	
MIB1						
< 10	44	9	NS	33	18	NS
> 10	8	3		5	6	
PCNA						
< 10	18	3	NS	16	3	< 0.0001
10-50	12	4		12	4	
> 50	22	5		10	17	
ER						
< 10	38	8	NS	27	16	NS
> 10	10	3		8	6	
PR						
< 10	40	8	NS	29	17	NS
> 10	10	4		8	6	



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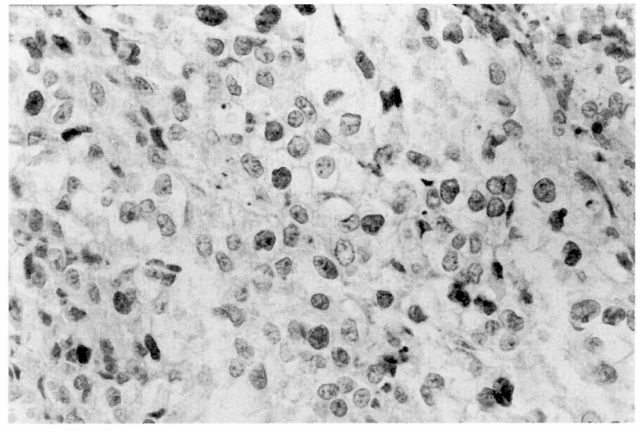

Fig. 2

Figure 1. — Immunohistochemical staining of cyclin D1 in endometrial carcinoma (x 400).
Figure 2. — Immunohistochemical staining of cyclin E in endometrial carcinoma (x 400).

Cyclin E expression in hyperplastic lesions. In hyperplasias, positive expression of cyclin E was found in 16/31 (51.6%) of the cases. A higher cyclin E expression in hyperplastic cases compared with those of normal proliferative phase of the endometrium was found ($p = 0.02$). A statistically significant relationship of cyclin E and progesterone was detected ($p = 0.027$) in this phase.

Cyclin E expression in endometrial carcinomas. Absence to low cyclin E expression (< 5% positive tumor cells) was found in 39/64 (60.9%), focal to moderately positive (5-50% positive tumor cells) in 19/64 (29.7%) and strongly positive (> 50%) in 6/64 (9.4%) of the carcinomas (Figure 2). Decreased cyclin E expression in endometrial carcinomas compared with the cases of hyperplasia ($p = 0.017$) was observed. The relationship between cyclin E expression with FIGO stage, vessel invasion, squamous metaplasia, menopausal status and steroid hormones was found to be statistically insignificant. A statistically significant positive correlation of cyclin E expression with p53 ($p = 0.052$), pRb ($p = 0.002$), PCNA ($p < 0.0001$) as well as with poorly differentiated carcinomas ($p = 0.096$) was found (Tables 2 and 3).

Discussion

Alterations in the expression of oncogenes or tumor suppressor genes (among them *c-erbB-2, c-myc, p53, Rb*) probably constitute the most frequent genetic alterations associated with the process of carcinogenesis [19]. The control of cell proliferation is one of the key issues in cancer progression. Proliferation and differentiation of endometrial cancer is regulated by various cell-cycle inhibiting factors. Our knowledge about these proteins and the mechanisms regulating cell-cycle progression has dramatically increased in recent years. Although the role of single cell-cycle regulators in endometrial cancer has been studied by several groups, there are only a few reports about the immunoexpression of cyclins D1 and E in normal, hyperplastic and neoplastic endometrium. In the present study, cyclin D1 immunoreactivity was restricted in a few cells of normal and hyperplastic endometrium. In endometrial carcinoma cyclin D1 was observed in all cases, but only 12/66 (18.2%) were overexpressed. However, in contrast with our study, Nikaido *et al.* [4] demonstrated that cyclin D1 expression was detected in 30/74 (40%) of endometrial cancers and diffuse positivity for cyclin D1 was associated with clinically advanced stages and advanced histological grades. Quddus *et al.*, found that overexpression of cyclin D1 was increased from normal endometrium to hyperplasia and carcinoma [20]. In our study there was no significant correlation between the cyclin D1 immunostaining pattern and clinicopathological parameters or with the markers, which is in agreement with the results of Ito *et al.* [14]. Although the role of cyclin D1 in carcinogenesis is not completely understood, several experimental observations suggest that this cyclin plays a key role in the development and progression of tumors. Wang *et al.* [21] found overexpression of cyclin D1 in transgenic mice with mammary tumors. Fantl *et al.* [22] found that amplification of cyclin D1 is present in 15% of primary human breast cancers, and Gillet *et al.* [8] reported that one-third of human breast carcinomas contain excessive levels of cyclin D1.

In the present investigation, no correlation was found between overexpression of cyclin D1 with PR-ER expression in endometrial carcinoma, which is considered to be a sex-steroid dependent tissue, and immunohistochemical analysis showed expression of hormone receptors in normal, hyperplastic and neoplastic endometrium. The negative expression of ER and PR in endometrial carcinoma has been found to be associated with higher tumor grade, advanced clinical stage and unfavorable patient prognosis [15, 23]. In normal endometrial cells, variations in the expression of cell cycle regulatory proteins play an important role during the menstrual cycle. In an immunohistochemical study, Shiozawa *et al.* [24] described a significant up-regulation of cyclins D1, E, B1, Cdk4 and Cdk2 in endometrial glandular cells during the proliferative phase. Zurkerberg *et al.* [25] demonstrated that cyclin D1 gene and estrogen receptor expression are positively correlated in primary breast cancer, and that estrogen stimulation increases cyclin D1 gene expression in ER-positive cultured breast cell lines. The mechanism of development of carcinogenesis of the endometrium may differ from mammary gland tissue. Overexpression of cyclin D1 expression has also been found in many types of tumors like squamous cell carcinoma of the uterine cervix [26], ovarian carcinomas [6, 27], transitional cell carcinoma of the urinary bladder [28], and head and neck squamous cell carcinoma [29].

Cyclin D1 (PRAD-1, CCND-1) is a part of the molecular system regulating the progression of the cell cycle from the G1 to S transition point, interacting with other molecules including the retinoblastoma gene product, by the mechanism of phosphorylation [30]. For this reason, we evaluated the correlation between the expression of this cyclin with the proliferative activity of the same tumors. We failed to demonstrate any correlation between these two molecules. Experimental studies on cell lines have shown that cyclin D1 is not required for the progression through G1 in the absence of functional pRb, indicating that at least in these cells pRb is the major and possibly the only target of cyclin D1 [31]. Alterations of the Rb gene have been observed in several epithelial tumors suggesting that structural abnormalities, including mutations and/or deletions of the Rb gene, may result in the activation of tumor suppressor proteins and may be involved in tumorigenesis. Jiang *et al.* [32] in their study concluded that the inhibitory effect of Rb on cell cycle progression can be abrogated during tumor development either by loss of expression of the Rb gene or by increased expression of the cyclin D1 gene in human esophageal cancer.

Alterations of the human p53 gene, either in the form of gene loss or mutations, have been observed to be a common change in endometrial carcinomas related to tumor development and progression [16, 33]. Immunohistochemical overexpression of p53 has also been reported to be correlated with high grade and advanced tumors, as well

as, with decreased patient survival [33]. In the current study, the absence of p53 expression in either normal or hyperplastic endometrium is comparable to the findings of previous studies [23, 33]. Moreover, no correlation was found between cyclin D1 over-expression and p53 expression in endometrial carcinomas, which is in accordance with the findings of other investigators, suggesting the lack of a p53-cyclin D1 pathway. The endometrium is an actively proliferating tissue and there is an overlap in the cell proliferation fraction between benign endometrium and endometrial carcinoma. Determination of the MIB1 (Ki67) index in a series of normal, hyperplastic and malignant lesions showed no evidence of correlation with progression to malignancy [23]. High PCNA indices have been associated with advanced cancer stage, myometrial invasion and c-erbB-2 expression [34]. In our study, we failed to demonstrate any correlation between the immunoreactivity of cyclin D1 with the proliferative markers PCNA and MIB1.

In the current study cyclin E immunoreactivity was detected in 39.1% of the endometrial carcinomas examined, in agreement with the study of Milde-Langosch *et al.* [3]. Previous reports demonstrated that a higher cyclin E was expressed in cancers of the uterine cervix and ovary than in normal cervical epithelium and the ovary [35, 36]. In the present study, we demonstrated that cyclin E expression is significantly increased with histological grades of endometrial carcinoma. Previous reports similarly showed that increased cyclin E expression was linked with histological grade in endometrial carcinoma and breast cancer [37-39]. On the other hand, Ito *et al.* [14] reported that the expression of cyclin E was not correlated with histological grade in endometrial carcinoma. In our study we found a positive correlation between the expression of cyclin E and p53 ($p = 0.052$) in endometrial carcinoma. Li *et al.* [40] reported that cyclin E expression was detected in all 17 (30.4%) cases of human endometrial cancer which were positive for p53 over-expression, and the pattern of cyclin E expression was similar to that of p53. Over-expression of p53 is known to occur more frequently in poorly differentiated carcinomas with p53 mutations [16]. Therefore, elevated expression of cyclin E is a characteristic feature of high-grade endometrial carcinomas associated with p53 expression. Mutated p53 is considered to be unable to bind the promoter of cyclin E and might allow uncontrolled expression of cyclin E mRNA [40]. Thus, the correlation between the expression of cyclin E and p53 in endometrial carcinomas might represent the possible interactions of these molecules [42]. Keyomarsi *et al.* [37] found that the expression of cyclin E protein occurred in most of the breast tumors tissue examined, and the alterations increased with increasing grade and stage of the tumor, suggesting that it can be used as a prognostic marker in breast cancer. The alteration of cyclin E becomes more severe with breast tumor stage and grade and is more consistent than in cell proliferation or other tumor cell cycle related markers, such as cyclin D1 [37, 38]. PRb expression was up-regulated in endometrial carcinomas relative to normal endometrial tissue samples. This is in accordance with immunohistochemical studies which showed weak or absent Rb expression in normal endometrial cells, but strong Rb immunoreactivity in most endometrial hyperplasias and carcinomas [18]. Furthermore, mutations or re-arrangement of the Rb tumor suppressor gene, which are involved in carcinogenesis of many human tumors, may be rare events in endometrial carcinomas [41]. In contrast to cyclin D1, the requirement for cyclin E is independent of the pRb pathway [42]. Lack of retinoblastoma suppression function may therefore play a role in the development of endometrioid type endometrial cancer. On the other hand, cyclin E, but not cyclin D1 is essential for the G1/S transition in cells lacking pRB function [43, 44].

In conclusion, in the development of endometrial cancer, the abnormal expression of steroid receptors, tumor suppressor gene products and cyclins seem to exist, and could be the cause of advancement of malignancy. Taken together, our data indicate that cyclin E may play an important role in the regulation of differentiation and proliferation in endometrial cancer, and that cyclin D1 over-expression does not seem to play a pivotal role in the development and/or progression of human endometrioid endometrial carcinoma. Future studies should delineate the mechanism of these alterations in tumor cells and investigate the consequences of cyclin E alterations in the transformation process.

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