

Dp103 in Egyptian Patients with Breast Cancer: Prognosis to Lymph Node Metastasis

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Authors' contributions

This work was carried out in collaboration between all authors. Author HGAEA designed the study. Authors HGAEA and SSES wrote the protocol and wrote the first draft of the manuscript. Authors HGAEA, SSES and DMAEL performed the practical work, performed and managed the statistical analysis of the study and managed the literature searches. Authors MG and MMK collected the samples and did the pathological analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background: Dp103 is a multifunctional protein which binds and unwinds RNA secondary structures, therefore plays a vital role in RNA metabolism from birth to death.

Aims: The present study aimed to evaluate both serum levels and tissue expressions of Dp103 and explored their possible roles in lymph node metastasis within breast cancer Egyptian patients.

Methodology: Sixty patients were enrolled in this study; they were subdivided into two groups. First group included forty females newly diagnosed as primary breast cancer their age ranged from 26 to 81 and further subdivided in two groups according to lymph node involvement into twenty two patients without metastatic lymph node and eighteen patients with metastatic lymph node. Second group involved twenty females newly diagnosed as benign breast tumor their age ranged from 32 to 65, in addition to eighteen apparently healthy females aged range from 28 to 65 as controls. Serum level of both Dp103 and MMP-9 were determined using ELISA technique. CA 15.3 and

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CEA were determined using available commercial kits. Expression level for both Dp103 and NF- κ B were estimated using qPCR.

Results: Serum levels of both Dp103 and MMP-9 were significantly higher in primary breast cancer patients when compared to benign tumor and healthy females ($p < 0.05$). As well as, the expression levels of both Dp103 and NF- κ B were significantly high in metastatic lymph node when compared to benign tumor and none metastatic lymph node groups ($p < 0.05$). Also, serum levels of both Dp 103 and MMP-9 showed the same pattern of accretion when compared to none metastatic lymph node. Significant positive correlation was found between serum Dp103 in primary breast cancer patients and CA15.3 ($r = 0.3195$ and $p = 0.044$).

Conclusion: Dp103 might be used as a prognostic biomarker for breast cancer progression and metastasis.

Keywords: Dp103; MMP-9; NF- κ B; qPCR; breast cancer.

1. INTRODUCTION

Breast carcinoma is considered one of the most predominant malignancies affect women around the world. Mortality from breast cancer is almost due to invasion and metastasis of carcinomic cells to distant organ sites [1-2]; subsequently, identifying genes implicated in metastasis of breast cancer is of great importance. Since it is difficult to precisely predict the risk of metastasis in carcinomic patients, at the moment, more than 80% of them receive adjuvant chemotherapy, but almost 40% of the patients suffer relapse and at the end die from metastatic disease. Metastasis is a multistage procedure by which cancer extended from the place at which it first arose as a primary tumor to other organs in the body. These stages are interconnected over a chain of adhesive interactions and invasive processes, involving tumor angiogenesis, invasion and colonization [3-4]. Mechanisms involving metastasis remain poorly understood, due to its heterogeneity and nature. Matrix metalloproteinase-9 (MMP-9) is a zinc-dependent peptidase which belongs to gelatinase subfamily. As an inactive pro-enzyme, MMP-9 is excreted and undergoes activation by distinct sorts of extracellular proteases [5] and its activity is regulated by various biochemical stimulators such as growth factors and cytokines [6]. MMP-9 has been engaged in various processes related to tumor invasion; tumor-induced angiogenesis and tumor microenvironment modulate immunity [7]. High levels of MMP-9 expression are associated with invasion, metastasis and poor prognosis of several cancer types as cervical [8], colorectal [9], ovarian [10] and breast cancer [11]. Moreover, increase serum and urine levels of MMP-9 have also been found to be correlated with metastasis and poor prognosis in a variety of cancers [12]. Nuclear Factor-kappa B (NF- κ B) is a regulated transcription factor which

composed of homo or heterodimers of five REL proteins which are; NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB, and c-Rel (Rel). In non-stimulated cells, NF- κ B is being bound to its regulatory protein inhibitor of NF- κ B (I κ B). Phosphorylation of I κ B, leads to dissociation of the NF- κ B:I κ B complex and NF- κ B activation and localization of it from cytoplasm to the nucleus [13]. After homo or heterodimer formation, NF- κ B binds to special promoter sequences within many genes, which play vital roles in cell growth and apoptosis [14]. NF- κ B and its target genes have been involved in all cancer hallmarks [15]. Activation of NF- κ B has been demonstrated in cell lines of lung [16], breast [17], lymphoma [18], and leukemia [19]. Inhibition of NF- κ B signaling or gene knock out has been shown to mediate anti-tumor responses [20]. DEAD-box protein-103 (DP103) belongs to the family DExD/H-box RNA that contains a highly-conserved Asp-Glu-Ala-Asp/His motif within the helicase domain. DP103 was initially identified as a component of motor neuron molecule (SMN) both with Sm ribonucleoproteins and other Gemin proteins [21]. DP103 is a transcriptional repressor for Egr2 in hind brain development [22] and constitutes a repressor complex with METS-PE-1 to silence transcription of Ets target genes which are entangled in Ras-dependent macrophage proliferation and differentiation [23]. The current study aimed to estimate both levels of serum and tissue expression of Dp103 and explore their possible correlation with lymph node metastasis in breast cancer Egyptian patients.

2. METHODOLOGY

2.1 Ethical Approval

The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University and was conducted according to the rules of

Helsinki declaration for human studies. A Written informed consent was obtained from all study subjects.

2.2 Subjects

This study included an overall number of 60 patients; they were divided into two groups; the first group were 40 patients with invasive ductal carcinoma without any clinically features of distant metastasis, they were subdivided in accordance to lymph node involvement into 22 patients without metastatic lymph node and 18 patients with metastatic lymph node, the second group contained 20 patients with benign breast tumor and both groups were newly diagnosed at National Cancer Institute, Cairo, Egypt. In addition to 18 apparently healthy female blood donors are taken as a control group for serum analysis procedures. The classification of tumor and its stage were performed according to the international union against cancer (Tumor–Node–Metastasis) classification. The breast cancer histopathology was carried out in all cases with tissue biopsy from tumor cancer tissues (the majority was invasive ductal carcinoma). The pre-treatment staging procedures included physical and blood examinations, mammography, mammary ultrasound scanning, breast core biopsies, and chest X-rays. Also, the benign breast tumor histopathology was implemented in all cases by tissue biopsy of mammary tumor or after surgery. Furthermore, three tissue cores were taken from all breast lesions under ultrasound tutelage during biopsy set by 14 G true cut automatic biopsy needle, one of them stored in RNA lysis solution at -800C before genetic processing of Dp 103 and Nf-κB genes and other two cores stored within formalin 10% for histopathological and hormonal receptors assessment.

2.3 Biochemical Analysis

Five ml of peripheral blood sample were collected for serum separation and determination of Dp103 (ELISAKitassays.com, Catalog No.

ELISAHu005596) and MMP-9 (Cusabio kits, Catalog No.CSB-E08006h) using ELIZA technique. Also, cancer antigen 15.3 (CA 15.3) and carcinoembryonic antigen (CEA) were assessed following standard laboratory methods.

2.4 Real Time PCR

Expression of mRNA was determined using real time polymerase chain reaction. Primers sets for each gene are listed in (Table 1). Total RNA was extracted from benign, breast cancer and adjacent healthy breast tissue as control groups using RNeasy Mini kit (QIAGEN, Germany) as specified by the manufacture protocol. RNA purity was verified spectrophotometrically at 260/280 nm. Equal amounts of RNA (2µg) were reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (QIAGEN, Germany). PCR amplification using Step One thermal cycler (applied biochemistry) was fulfilled in 20 µl reaction mixture consisting of 10 µl SYBR® Green PCR Master Mix (cat. No. 204143 or 204163), 1 µl forward primer (nM), 1 µl reverse primer (nM), 3 µl cDNA, and 5 µl RNase free water. The following program was used for Dp103: initial heat activation for 15 min. at 95 C0, denaturation for 15 sec. at 94 C0, annealing for 30 sec. at 60 C0 and extension for 30 sec. at 72 C0 and number of cycles were 40 cycles while for NF-κB was 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The sequences of PCR primer pairs utilized for each gene are shown below. All values were normalized to the GAPDH genes as an invariant endogenous control (reference gene). The relative expression was calculated from the 2-ΔΔCT formula [24].

2.5 Statistical Analysis

Data were assessed with Graph Pad prism software using Student *t* test and Pearson's test. Results were expressed as means ± standard deviation and *p* < 0.05 was considered statistically significant.

Table 1. List of primers sequences applied in real time-PCR

Genes	Forward primer	Reverse primer
GAPDH	ATTCCACCCATGGCAAATTC	TGGGATTTCCATTGATGACAAG
NF-κB	GTGGTGCCTCACTGCTAACT	GTGGTGCCTCACTGCTAACT
Dp 103	GGAAGGCTTAGAGTGTTCATGTC	TGAGTTGCTTAATTCTGCCAGG

3. RESULTS

3.1 Patient Features

The study consisted of 60 patients with primary breast cancer aged between 28-81 years (54.89±13.04 years); subdivided into 22 patients without metastatic lymph node and 18 patients with metastatic lymph node, 20 patients with benign breast tumor aged between 32-65 years (43.25±11.18 years). All breast cancer types were invasive ductal carcinoma and the pathology of the benign tumor group was mainly fibroadenoma. In addition to, 18 healthy female as control group aged between 28-58 years (42.22±11.98 years). The clinocopathological characteristics of both benign and breast cancer patients listed in (Table 2).

3.2 Serum Levels of Tumor Markers

The levels of CA 15.3 and CEA in benign tumor group were significantly lower (17.14±6.19 and 2.22±0.87 respectively, $P < 0.001$) than in breast cancer group (104.70±11.40 and 14.0±20.39 respectively, $P < 0.001$) (Table 3).

3.2.1 Serum levels of Dp 103 and MMP-9

Serum level of Dp103 was significantly higher in breast cancer (26.58±7.55) when compared to control and benign tumor groups (18.14±2.83 and 19.42±4.38, respectively; $P < 0.001$), as well as, the serum level of MMP-9 in breast cancer group (700.17±184.01) was significantly higher than control and benign tumor groups (271.61±108.11 and 301±98.14, respectively, $P < 0.001$) (Table 3).

3.3 Tissue levels of Dp 103 and NF-κB

The expression levels of both Dp103 and NF-κB were significantly higher in breast cancer with positive lymph node than benign tumor tissues and none metastatic lymph node ($p < 0.001$) (Table 3). As well as, the serum levels of both MMP-9 and Dp 103 showed the same pattern when compared to none metastatic lymph node (Table 4).

3.4 Pearson's Correlation Analysis

There was a significant positive association between serum levels of Dp103 in primary breast cancer patients and CA 15.3 ($r = 0.3195$ and $P = 0.044$) (Fig. 1). There were no remarkable

significant correlations detected with any of the studied parameters and fold expression of Dp 103.

Table 2. Clinical examination and histopathological characteristics of primary breast cancer and benign breast tumour groups

Parameter	Percentage (%)
Grade	
Grade I	5.6
Grade II	75.0
Grade III	19.4
ER	
(Negative)	22.22
(Positive)	77.77
PR	
(Negative)	24.24
(Positive)	75.75
HER2	
(Negative)	70.58
(Positive)	29.41
Nodal status	
N0	55
N1	30
N2	7.5
N3	7.5
Tumor size	
T2	48.5
T3	42.4
≥T4	9.1
Benign breast tumor diagnosis	Percentage of patients
FA	70
FM	10
SCT	5
GM	5
FN	5
FET	5

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2. FA: Fibroadenoma, FM: Fibrocystic mastopathy, SCT: Spindle cell tumour, GM: Granulomatous mastitis, FN: Fat necrosis, FET: Fibro-epithelial tumour

3.5 Roc Analyses in Lymph Node Groups

The overall performance of serum Dp 103, MMP-9, and tissue expression of both Dp103 and NF-κB were assessed by ROC curve analysis. The best cut-off value for serum Dp103 in metastatic lymph node group was >24.6 ($P < 0.0001$) with 88.9% sensitivity and 63.6% specificity producing area under the curve (AUC) 0.792 (Fig.2). For tissue expression of Dp 103 in metastatic lymph node; the best cut-off point was >9.75 ($P < 0.001$) with 77.78% sensitivity and 100% specificity producing AUC= 0.896 (Fig. 3).

On the other hands, the best cut-off point for serum MMP-9 was >700 ($P < 0.001$) with 72.2% sensitivity and 81.8% specificity and AUC= 0.842 (Fig. 4), whilst for NF- κ B tissue expression, the best cut-off value was >17.27 ($P < 0.001$) with 77.8% sensitivity and 86.4% specificity producing AUC= 0.886 (Fig. 5).

Table 3. Age and biochemical parameters of the studied groups as mean \pm SD

Parameters	Control n=18	Benign breast tumor n=20	Primary breast cancer n=40
Age	42.22 \pm 11.98	43.25 \pm 11.18	54.89 \pm 13.04 ^{ab}
CA 15.3 (U/ml)	8.58 \pm 3.29	17.14 \pm 6.19	104.70 \pm 11.40 ^{ab}
CEA (ng/ml)	0.93 \pm 0.38	2.22 \pm 0.87 ^a	14.0 \pm 20.39 ^{ab}
Serum MMP-9 (ng/ml)	271.61 \pm 108.11	301 \pm 98.13	700.17 \pm 184.01 ^{ab}
Serum Dp103 (ng/ml)	18.14 \pm 2.84	19.42 \pm 4.38	26.58 \pm 7.55 ^{ab}

Significance is considered at $P < 0.05$. ^a P : significant vs. control group, ^b P : significant vs. benign breast tumor group

Table 4. Fold expression of Dp103 and Nf- κ B and serum levels of Dp 103 and MMP-9 in studied groups as mean \pm SD

Parameters	Adjacent normal breast tissue N=10	Benign breast tumor N=20	Adjacent normal breast tissue N=10	Primary breast cancer N=40	
				None metastatic lymph node N =22	Metastatic lymph node N =18
Fold expression of Dp103	1.06 \pm 0.19	1.73 \pm 0.13	1.01 \pm 0.12	7.03 \pm 1.91	13.79 \pm 4.79 ^{ab}
Fold expression of NF- κ B	1.33 \pm 0.26	5.64 \pm 1.68	1.00 \pm 0.00	14.02 \pm 3.86	22.18 \pm 7.80 ^{ab}
Serum level of Dp 103 (ng/ml)	-----	-----	-----	23.73 \pm 7.15	30.60 \pm 4.99 ^b
Serum level of MMP-9 (ng/ml)	-----	-----	-----	598.48 \pm 137.73	818.83 \pm 166.37 ^b

Significance is considered at $P < 0.05$. ^a P : significant vs. benign tumor group, ^b P : significant vs. none metastatic lymph node

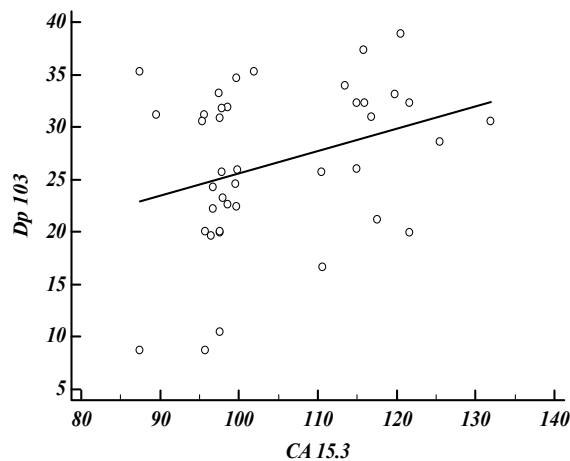


Fig. 1. Pearson’s correlation between serum levels of Dp 103 and CA 15.3 in primary breast cancer group ($r = 0.3195$ and $P = 0.044$)

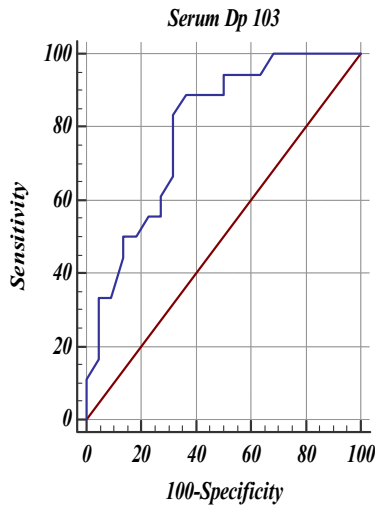


Fig. 2. ROC analysis of serum Dp 103 in metastatic lymph node

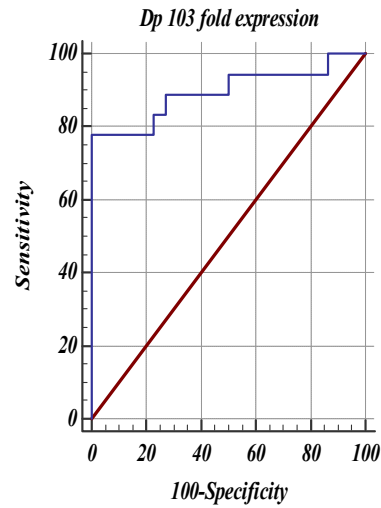


Fig 3. ROC analysis of Dp 103 expression in metastatic lymph node

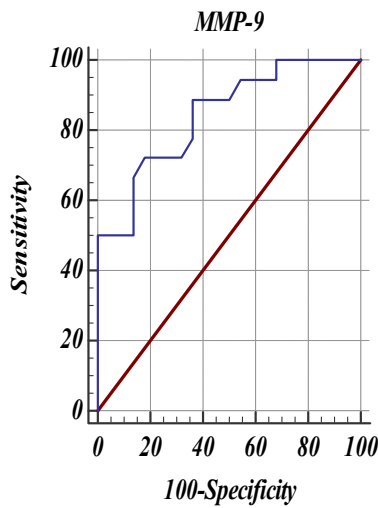


Fig. 4. ROC analysis of serum MMP-9 in metastatic lymph node

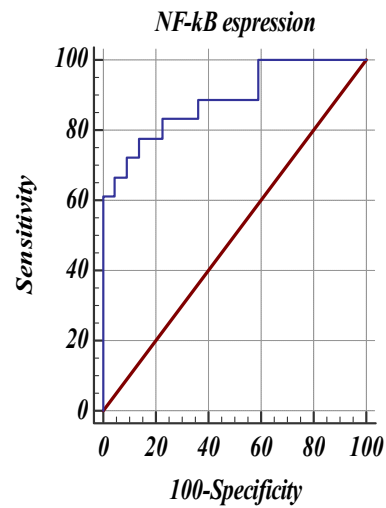


Fig. 5. ROC analysis of NF-κB expression in metastatic lymph node

4. DISCUSSION

Worldwide, breast cancer is considered one of the most common types of cancer affecting women [25]. Breast cancer is classified into two major types, ductal carcinoma (that starts in the cells of milk ducts) and lobular carcinoma (that starts in the breast lobules, the milk producing glands), and further divided into invasive (spread) or non-invasive (in situ) types [26]. Annually, it is estimated that more than one

million new cases of breast cancer are diagnosed [27]. As a consequence it is worthy to find more reliable biomarkers to provide early diagnosis and well prognosis of this type of cancer. The current study described for the first time the estimation of serum Dp103 level in breast cancer patients and we noticed that serum level of Dp103 was higher in primary breast cancer when compared to the control and benign tumor patient groups. Also, its level positively correlated with CA 15.3. Consequently, we could

not confirm our findings by other publications as there are not any available reports. We found that, serum levels of MMP-9 and CA 15.3 were higher in primary breast cancer patients than in benign tumor. Similar data were observed in the work of Ławicki et al., [28] who reported statistically significant higher plasma levels of VEGF, MMP-9, and CA 15-3 in patients with breast cancer compared with healthy women. Also, these results concur with Rashad et al., [29] who revealed that MMP-9 level was significantly higher in patients with positive lymph node than patients without lymph node involvement. Furthermore, Heo et al., [30] reported significant high serum levels of both MMP-2 and MMP-9 in breast cancer patients compared to patients with benign tumor. In experimental mouse models where lung carcinoma or melanoma cells injected into MMP9-deficient mice, manifested that MMP-9 in the injected mice promotes metastatic colonization of the lung [31]. Moreover, metastatic colonization was promoted in MMP-9 knockout mice by adding MMP-9 expressing bone marrow [31]; also MMP-9 enhances lung metastasis in multistage mammary tumor model, obtained from the pro-angiogenic participation of MMP-9 neutrophil in the lungs [32]. These studies support the idea that MMP-9 can reinforce metastasis expressed by stromal cells, at least in mouse models. However, different studies examining the expression of MMP-9 in human tumors using immunohistochemistry have concluded that MMP-9 is most excessively generated by tumor cells, with low expression frequency in fibroblasts and immune cells [33]. Regards to Dp103 tissue expression, we found that the expression in primary cancer was significantly higher than that of benign tumor and normal tissue. Upon subdivision of primary breast cancer patients according to lymph node involvement, we found that patients with metastatic lymph node had significantly elevated tissue expression of Dp 103 and NF-κB with serum level of Dp 103 and MMP-9 exhibited the same pattern comparing to patients with negative lymph node involvement, suggesting that Dp 103 and MMP-9 had a role in breast cancer metastasis. These result in agreement with Eun et al., [34], Heo et al., [30] and Daniele et al., [35]. In tumor cells, NF-κB is activated due to mutations in genes that encoding NF-κB transcription factors or in genes that control NF-κB activity. In addition, factors that influence NF-κB activity are secreted by some tumor cells [36]. Therefore, blocking NF-κB can stop proliferation of tumor cells or become more sensitive to anti-

tumor agents [37]. Cells with elevated NF-κB activity deregulate the production of chemokines, which increases migration activity [38]. In addition, NF-κB sites were specified in the promoters of genes which encode several proteolytic enzymes as matrix metalloproteinases (MMPs) that involved in promoting tumor invasion of surrounding tissue [39]. NF-κB has been involved in the development of cancers of epithelial origin, such as breast cancer [40]. Several studies have reported an elevation of NF-κB DNA-binding activity both in mammary carcinoma cell lines and primary breast cancer cells [41]. We found a higher expression level of NF-κB in primary breast cancer than both benign tumor and normal adjacent tissue. This was consistent with Eun et al., [34] who reported that activated NF-κB correlates with DP103 expression and patient survival. DP103 has ability on NF-κB regulation pathway and it may be crucial for its regulation of MMP9, as it regulates NF-κB-dependent gene expression due to multiple stimuli such as tumor necrosis factor alpha, interleukin- 1, and lipopolysaccharides, in addition to DNA damaging reagents, which initiate NF-κB activation pathway through binding to transforming growth factor activated kinase-1 (TAK1) and acting as a cofactor, thus increasing TAK1-mediated IKK2 phosphorylation, and hence NF-κB activation [34]. Knock down experiments of Dp103 showed down regulation of NF-κB activation by a wide range of general stimuli, as TNF-α and lipopolysaccharide, pointing to involvement of the IKKs and TAK1 which are fellows of the mitogen-activated protein kinase (MAPK). Using endogenous purified proteins, it was reported that DP103 can fairly bind to TAK1 and acts as a cofactor, thus boosting TAK1-mediated IKK2 phosphorylation, and hence NF-κB activation [34].

5. CONCLUSION

The present study is an exploratory survey as it, for the first time, studied serum Dp103 in Egyptian breast cancer women and revealed that MMP-9 serum level is associated with serum Dp103 and the last one may have a role in breast cancer metastasis and poor prognosis through activation of NF-κB pathway. The present study has a limitation: the sample size was relatively small. Consequently, the present study should be done on large number of breast cancer population to emphasize our results about serum

Dp103 and its prognostic effect on lymph node metastasis.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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