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Original Article

Determination of MMP-9 and Ki-67 Expression in Breast Carcinoma and Correlation of These Markers with Tumor Grade and Stage

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ABSTRACT

Objective: To evaluate the expression pattern of MMP 9 and Ki-67 in breast carcinoma and their correlation with tumor grade and pathological tumor stage(pT) using immunohistochemistry.
Materials and method: MMP-9 and Ki-67 expression in one hundred patients of primary breast carcinoma were assessed by immunohistochemistry at Nil Ratan Sircar Medical College and Hospital, Kolkata and scored as low expression and high expression. MMP-9 scoring 0 to 4 will be considered to represent low levels of expression while score from >4 to 12 will be considered as high levels of expression. Ki-67 scoring based on a count of at least 500 tumor cells. The Ki-67 values were expressed as the percentage of positive cells in each case. Cases with > 15% positive nuclei were classified as high Ki-67 expression, and those with < 15% were classified as low Ki-67 expression
Result: In our study MMP-9 showed high expression in 63% (63/100) cases and Ki-67 showed high expression in 65% (65/100) cases. Both the markers showed positive correlation with tumor grade and stage. (statistically significant at p<.05)
Conclusion: Hence MMP-9 and Ki-67 expression correlated strongly with higher tumor grade and stage indicating that these can be used as target for development of novel therapies.

Keywords: *Breast carcinoma, MMP-9, Ki-67, Immunohistochemistry.*

INTRODUCTION

Breast carcinoma is one of the most commonly occurring cancers in women globally, and one of the leading causes of mortality in Indian women. 6% to 10% of patients still present with metastatic breast cancer at the time of diagnosis; for those patients, relapses tend to occur earlier and survival rates are shortened [1]. Cancer metastasis is considered to develop in a step-wise fashion leading to the acquisition of new capabilities by tumor cells helping them to thrive and evade natural barriers [2]. Degradation of the extracellular matrix (ECM) is thought to be a crucial step in the formation of tumor metastasis. Multiple proteolytic enzymes such as plasmin, cathepsins, and matrix metalloproteinases (MMPs) are known to degrade ECM [3]. Matrix metalloproteinase-9 (MMP 9) is a zinc-dependent peptidase that belongs to the gelatinase subfamily of MMPs. It is excreted as an inactive pro-enzyme that undergoes activation upon cleavage by different types of extracellular proteases [4]. MMP 9 activity is thought to be regulated by different biochemical stimulators such as growth factors and cytokines whose expression appear to modulate intracellular signalling pathways [5]. MMP 9, also known as gelatinase B, plays an important role in extracellular matrix (ECM) remodelling, protein cleavage, and is associated with tumor invasion, metastasis and modulation of tumor microenvironment [6,7]. MMP 9 has the capability to degrade collagens, including Type IV collagen [8], which plays a role in basement membrane degradation promoting migration, invasion and metastases.

Uncontrolled proliferation is a distinct characteristic of malignancy and may be assessed through various methods, including counting mitotic figures in stained tissue sections, incorporation of labelled nucleotides into DNA, and flow cytometric evaluation of cell fraction in S phase [9]. However, the most common measurement involves immunohistochemical assessment of Ki-67 antigen. Ki-67 is present in all proliferating cells, and its role as a proliferation

marker attracts considerable interest. Ki-67 is a nuclear nonhistone protein present in all active phases of cell cycle, except the G_0 phase [10].

This current study, therefore, aims to examine the pattern of expression of MMP 9 and KI-67 and correlation of these markers with tumor grade and stage in diagnosed cases of primary breast carcinoma.

MATERIALS AND METHODS

A total of 100 cases of breast carcinoma were studied at the Department of Pathology, Nil Ratan Sircar Medical College and Hospital, Kolkata. The specimens were of modified radical mastectomy, and relevant history of the patients was taken (like age, prior chemotherapy etc.) The grossing and reporting of the specimens was done according to the College of American

Pathologists (CAP) Protocol for the examination of specimens from patients of invasive carcinoma of breast. The specimens were fixed in 10% neutral buffered formalin. Representative sections were taken, and after proper tissue processing, were embedded in paraffin, and stained with hematoxylin-eosin stain. Grading of breast carcinoma was done according to the Nottingham combined histologic grade (Elston-Ellis modification of Scarf-Bloom-Richardson grading system) and staging was done according to pathological TNM staging (WHO).

Immunohistochemistry for MMP 9 & Ki-67:

3.0 µm sections were taken on poly-L-lysine coated slides and were deparaffinized in xylene, followed by hydration in descending grades of ethanol. Antigen retrieval was performed by heating sections in microwave using TRIS-EDTA buffer pH 9.0 for both MMP 9 and Ki-67. Sections were then incubated with power block for 10 min to reduce non-specific antibody binding, followed by incubation with primary antibodies for 1 hr at 4ºC. Rabbit monoclonal antibody against MMP [Bio-SB, EP127-clone] & rabbit monoclonal antibody against Ki-67 [CELL MARQUE-sp6] were used. After three washes with trisphosphate buffer solution (TBS), secondary antibody was added and incubated for 30 min. After further three washes with TBS, 3,3'-diaminobenzidine substrate (DAB tetrahydrochloride) was applied to the sections for 10 minutes and the sections were counterstained with hematoxylin, dehydrated with ethanol and xylene and mounted permanently with DPX. Negative control sections were processed by omitting the primary antibody. Positive controls were: (1) Normal splenic tissue for MMP 9, (2) Normal tonsillar tissue for Ki-67.

Evaluation of Staining:

In this study, Scoring of MMP 9 expression on each resected specimen will be carried out using a two-tier scoring system. The first parameter corresponds to the percentage of immunoreactive cells also known as the quantity score (QS). QS will be estimated as follows (no staining was scored as 0, 1-10% of cells with positive staining will be scored as 1, >10- 50% as 2, >50-70% as 3, and >70-100% as 4). We will next assess the second parameter (staining intensity score), which will be rated as follows: No staining → 0, weak staining →1, moderate staining → 2, and strong staining → 3. The product of the quantity and the staining intensity scores represents the total IHC score that ranges from 0 to 12. IHC scores of 0 to 4 will be considered to represent low levels of expression while score from >4 to 12 will be considered as high levels of expression (Table No. 1,2,3).

Ki-67 scoring based on a count of at least 500 tumor cells. The Ki-67 values were expressed as the percentage of positive cells in each case. Cases with > 15% positive nuclei were classified as high Ki-67 expression, and those with < 15% were classified as low Ki-67 expression (Table No. 4).

Statistical Analysis:

Chi-square test was performed to determine the correlation between MMP 9 & Ki-67 expression with tumour grade and pathological tumor stage. p-value of <0.05 was considered statistically significant.

RESULTS

In our study 15 cases belong to 40-49 years age group and 66 case belong to 50-59 years age group.19 cases are of more than 60 years (Table No. 5). Mean age of the patients was 55 years (ranging from 44 to 69 years). The majority of the cases were infiltrating ductal carcinoma, not otherwise specified (IDC NOS), followed by invasive breast carcinoma of no special type (IBC, NST), which were confirmed by hematoxylin-eosin staining. Elston-Ellis modification of Scarf- Bloom Richardson grading was carried out on all cases, and most patients belonged to grade 3 (66/100, 66.0%), followed by grade 2 (34/100, 34%) (Table No. 6). MMP 9 &Ki-67 immunostaining was done in all 100 cases and similarly staging was also carried out in all cases according to WHO TNM staging and most patients belongs to pT4 (36/100,36%), followed by pT3 (31/100, 31%), pT1 (17/100,17%), pT2 (16/100,16%) (Table No. 7).

MMP 9 showed high expression in 63 out of 100 cases (63%) & showed low expression in 37 cases (Table No. 8) and Ki-67 showed high expression in 65 out of 100 cases (65%) & showed low expression in 35 cases (Table No. 9).

Expression of MMP 9 and Ki-67 and their correlation with tumor grade and pathological tumor stage(pT)

A positive correlation was observed between MMP 9 expression and tumour grade at p<0.05 level. The percentage of high expression of MMP-9 increased from 23.5% [8 out of 34] in **grade 2** to 83.3% [55 out of 66] in **grade 3**. A positive correlation was observed between MMP 9 expression and pathological tumour stage(**pT**) at p<0.05 level.The percentage of high expression of MMP-9 increased from 29.4 % [5 out of 17] in **pT1** to 31.2% [5 out of 16] in **pT2** and 67.7% [21 out of 31] in **pT3** to 88.8% [32 out of 36] in **pT4**. A positive correlation was observed between Ki-67 expression and tumour grade at p<0.05 level. The percentage of high expression of Ki-67 increased from 35.2% [12 out of 34] in **grade 2** to 80.3% [53 out of 66] in **grade 3**. A positive correlation was observed between Ki-67 expression and pathological tumour stage(**pT**) at p<0.05 level The percentage of high expression of Ki-67 increased from 17.6 % [3 out of 17] in **pT1** to 43.7% [7 out of 16] in **pT2** and 80.6% [25 out of 31] in **pT3** to 83.3% [30 out of 36] in **pT4.**

TABLE NO.1 Quantity Score for MMP-9

SCORE	STAINING
0	No staining
1	1-10% of cells with positive staining
2	>10-50% of cells with positive staining
3	>50-70% of cells with positive staining
4	>70-100% of cells with positive staining

TABLE NO.2 Staining intensity for MMP-9

SCORE	STAINING
0	No staining
1	Weak staining
2	Moderate staining
3	Strong staining

TABLE NO.3 Total IHC Score for MMP-9

SCORE	EXPRESSION PATTERN
0-4	LOW
>4-12	HIGH

TABLE NO. 4 IHC Score for Ki-67 (Done on count of atleast 500 tumor cells)

% OF POSITIVE NUCLEI	EXPRESSION PATTERN
<15	LOW
>15	HIGH

TABLE NO. 5 Distribution of patient according to age

AGE	NO. OF PATIENTS
40-49	15
50-59	66
>60	19

TABLE NO. 6 Distribution of patient according to grade

GRADE	No. of patients
GRADE-2	34
GRADE-3	66

TABLE NO. 7 Distribution of patients according to stage(pT)

STAGE(pT)	No. of patients
T1	17
T2	16
T3	31
T4	36

TABLE NO. 8 MMP-9 Expression In Breast Carcinoma

MMP-9 EXPRESSION	NO OF PATIENTS
HIGH	63
LOW	37

TABLE NO. 9 Ki-67 Expression In Breast Carcinoma

Ki-67 EXPRESSION	NO OF PATIENTS
HIGH	65
LOW	35

TABLE NO. 10 Correlation of MMP-9 expression with tumor grade

BREAST CARCINOMA	NO. OF PATIENTS	MMP-9 EXPRESSION			
		HIGH	LOW	Chi square	p-value*
GRADE-2	34	8	26	34.4302	<0.00001
GRADE-3	66	55	11		

***Significant**

TABLE NO. 11 Correlation of MMP-9 expression with tumor stage

BREAST CARCINOMA	NO. OF PATIENTS	MMP-9 EXPRESSION			
		HIGH	LOW	Chi square	p-value*
pT1	17	5	12	25.7973	0.000011
pT2	16	5	11		
pT3	31	21	10		
pT4	36	32	4		

***Significant**

TABLE NO. 12 Correlation of Ki-67 expression with tumor grade

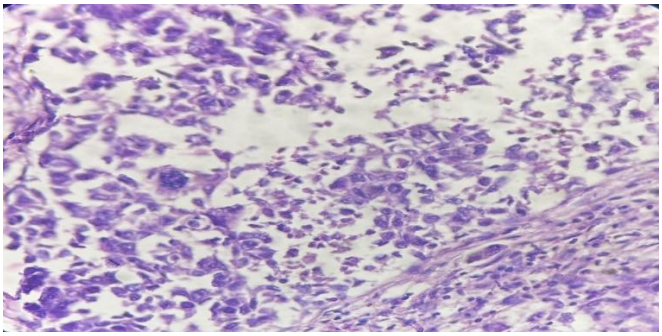
BREAST CARCINOMA	NO. OF PATIENTS	Ki-67 EXPRESSION			
		HIGH	LOW	Chi square	p-value*
GRADE-2	34	12	22	19.982	<0.00001
GRADE-3	66	53	13		

***Significant**

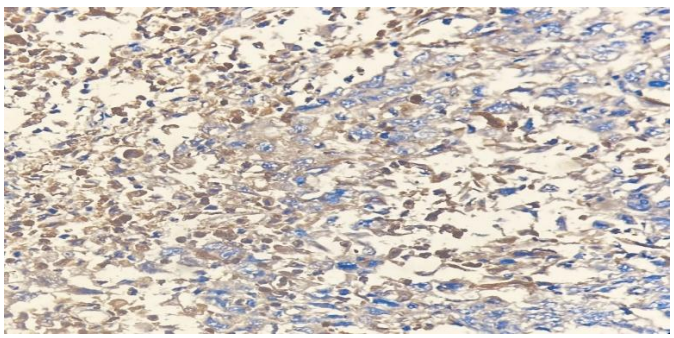
TABLE NO. 13 Correlation of Ki-67 expression with tumor stage

BREAST CARCINOMA	NO. OF PATIENTS	MMP-9 EXPRESSION			
		HIGH	LOW	Chi square	p-value*
pT1	17	3	14	28.5855	<0.00001
pT2	16	7	9		
pT3	31	25	6		
pT4	36	30	6		

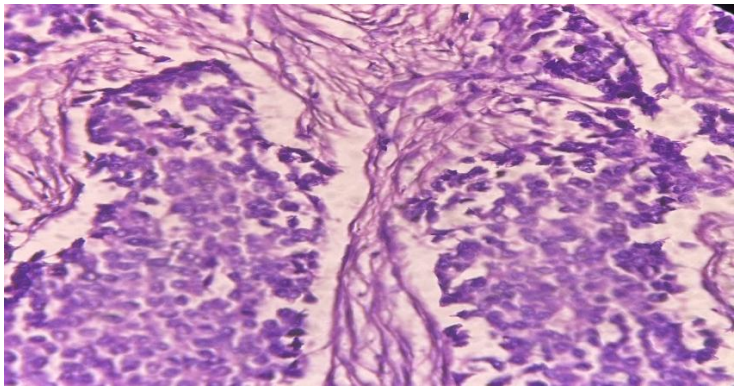
***Significant**



HEMATOXYLIN &EOSIN STAIN SECTION SHOWING BREAST CARCINOMA



BREAST CARCINOMA SHOWING HIGH MMP-9 EXPRESSION



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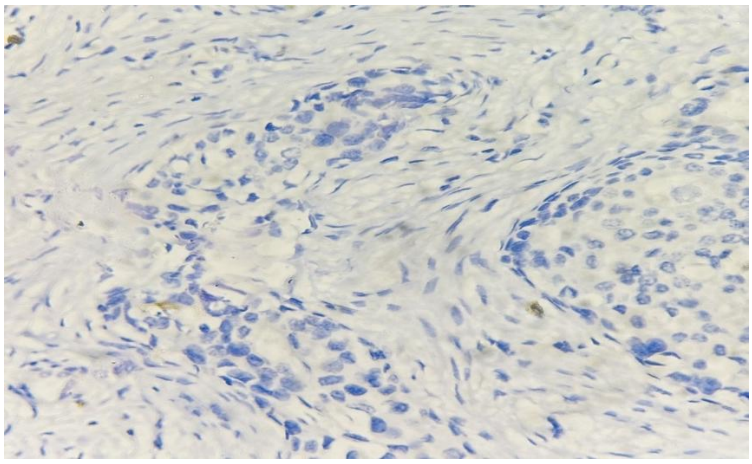
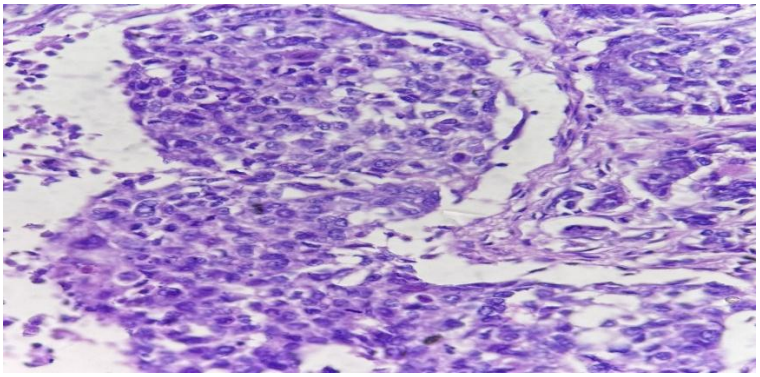
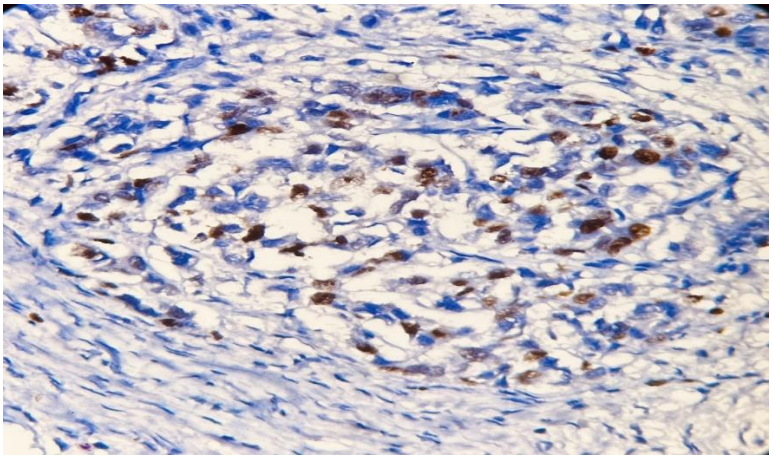


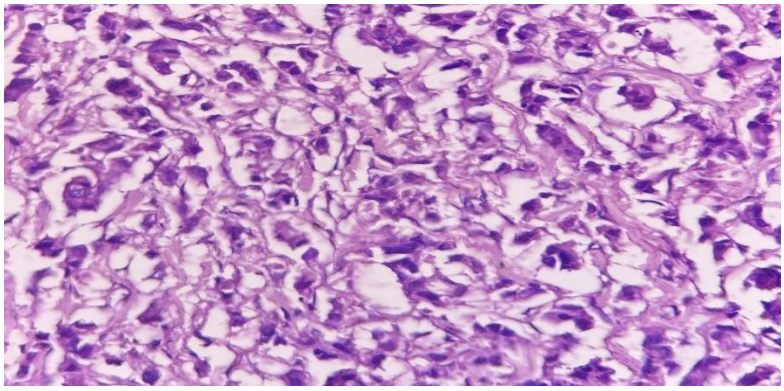
BREAST CARCINOMA SHOWING LOW MMP-9 EXPRESSION [NEGATIVE STAINING]



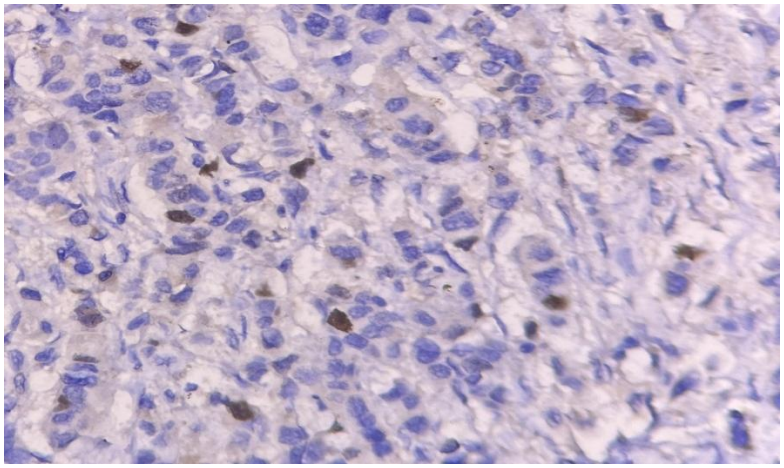
HEMATOXYLIN &EOSIN STAIN SECTION SHOWING BREAST CARCINOMA



BREAST CARCINOMA SHOWING HIGH Ki-67 EXPRESSION



HEMATOXYLIN &EOSIN STAIN SECTION SHOWING BREAST CARCINOMA



BREAST CARCINOMA SHOWING LOW Ki-67 EXPRESSION

DISCUSSION

Yousef *et al.* found 60.5% [121 out of 200] patients have high MMP-9 expression and 39.5% [79 out of 200] patients have low expression. In our study we have found 63% patients have high MMP-9 expression and 37% patients have low expression [11].

Yousef *et al.* found that there is a positive correlation between elevated levels of MMP-9 and breast cancer and high histological grade. Their study confirmed that there is association between the high levels of MMP-9 expression with tumors of high histological grade (Grade III) that is high grade of MMP-9 expression were found in 79.4% of Grade III cases [11].

Also, **McGowan *et al.*** shows a significant correlation between the expression of MMP-9 and tumor grade [12]
In our study we have found that high MMP-9 expression was found in 83% cases of Grade III cases.

In another study **Merdad *et al.*** found 55% cases with high expression of MMP-9[13]. In our study we have found 63% cases with high expression of MMP-9.

Merdad *et al.* found very significant correlation between tumor grade and MMP-9 expression. The percentage of high MMP-9 expression increased in 31.8% [7 out of 22]in histological Grade 1 to 59.3% [57 out of 96] in histological Grade 2 and 3[13].

In our study we have found a positive correlation was observed between MMP 9 expression and tumour grade at $p<0.05$ level. The percentage of high expression of MMP-9 increased from 23.5% [8 out of 34] in **grade 2** to 83.3% [55 out of 66] in **grade 3.**

In our study we have found a positive correlation was observed between MMP 9 expression and pathological tumour stage(**pT**) at $p<0.05$ level.The percentage of high expression of MMP-9 increased from 29.4 % [5 out of 17] in **pT1** to 31.2% [5 out of 16] in **pT2** and 67.7% [21 out of 31] in **pT3** to 88.8% [32 out of 36] in **pT4**. In support to our study **Zhang *et al.*** [14] had found significant correlation between pathological tumor staging(**pT**) i.e. the larger tumor size with high expression of MMP-9 In contrary **Pellikainen *et al.*** [15] & **Scorilas *et al.*** [16] exclusively reported the significant correlation between high MMP-9 expression with smaller tumor size (pT).

Viale *et al.* [17] had found that in their study high Ki-67 was associated with larger tumors i.e. pathological tumor stage, higher tumor grade. According to **Viale *et al.*** [17] the percentage of high Ki-67 expression increased in 25.4% [192 out of 753]in histological Grade 1 to 49.1% [632 out of 1287] in histological Grade 2 and 78.5% [326 out of 415] in histological Grade 3

Soliman *et al.* [18] had found high Ki-67 expression (> 15%) was present in 36 cases (33%). In our study we got in 65% cases with high Ki-67 expression. **Soliman *et al.*** [18] found very significant correlation between tumor grade andKi-67 expression. The percentage of high Ki-67 expression increased in 15.7% [6 out of 38]in histological Grade 1 to 31.1% [14 out of 45] in histological Grade 2 and 66.6% [16 out of 24] in histological Grade 3

In our study we have found very significant correlation between tumor grade and Ki-67 expression ($p < 0.05$). The percentage of high Ki-67 expression increased in 35.2% [12 out of 34]in histological Grade 2 to 80.3% [53 out of 66] in histological Grade 3.

In our study we have found a positive correlation was observed between Ki-67 expression and pathological tumour stage(**pT**) at $p<0.05$ level. The percentage of high expression of Ki-67 increased from 17.6% [3 out of 17] in **pT1** to 43.7% [7 out of 16] in **pT2** and 80.6% [25 out of 31] in **pT3** to 83.3% [30 out of 36] in **pT4**. In support to our study **Viale *et al.*** had found significant correlation between high Ki-67 expression with larger tumor size i.e. high pathological tumor stage (**pT**). The percentage of high expression of Ki-67 increased from 42.5 % in **pT1** to 54.2% in >**pT1.** In contrary **Soliman *et al*** had found negative correlation of high expression of Ki-67 with tumor size. The percentage of high expression of Ki-67 decreased from 40 % in **pT1** to 33.3% in >**pT1.**

CONCLUSION

From our study it can be concluded that MMP-9 and Ki-67 has significant correlation with tumor grade and tumor stage. So, they can be used as a marker of poor prognosis. They can be used as target for the development of novel therapy.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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