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A Study of Immunohistochemistry Profiling of Matrix Metalloproteinase-9 and Interleukin-8 in Triple Negative Breast Cancer Patients

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Abstract

Introduction: Triple-negative breast cancer (TNBC) is a highly aggressive subtype characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2, limiting treatment primarily to chemotherapy, radiation, and emerging immunotherapies. It is associated with a poor prognosis, a high likelihood of metastasis, and resistance to chemotherapy. Matrix metalloproteinase-9 (MMP-9) and interleukin-8 (IL-8) are key contributors to TNBC progression—MMP-9 facilitates tumor invasion and blood vessel formation, while IL-8 enhances cancer cell survival, migration, and metastasis through inflammatory pathways. Increased levels of these biomarkers are linked to more aggressive disease and worse survival outcomes. This study evaluates MMP-9 and IL-8 expression in breast cancer tissues using immunohistochemistry (IHC) and explores their association with clinicopathological features. **Methods:** A cross-sectional study was carried out at the NABL-accredited laboratory in the Department of Pathology, JSS Hospital, Mysuru, between January 2020 and May 2024. The study involved 29 paraffin blocks from patients who had undergone mastectomy or core biopsy following a breast carcinoma diagnosis. Hematoxylin and eosin (H&E) staining, as well as immunohistochemistry (IHC) staining, were performed and the findings documented. **Results:** The results demonstrated expression of IL-8 correlates with triple-negative breast carcinoma patients and may also serve as a prognostic biomarker for breast cancer progression. **Conclusion:** These observations imply a possible interaction between MMP-9 and IL-8 expression in breast cancer, where IL-8 positivity is increasingly seen in MMP-9 positive cases. The statistically significant reverse correlation between MMP-9 negative and IL-8 negative

cases could suggest that IL-8 expression is dependent on the presence of MMP-9. Further research is needed to clarify the mechanistic interaction and its prognostic influence on tumor development and prognosis.

Keywords: Matrix Metalloproteinase-9; Interleukin-8; Triple-Negative Breast Cancer; Tumor budding; Km Score; Ki 67

1 Introduction

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer, distinguished by the absence of three key receptors commonly found in other types of breast cancer: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).[1] These receptors drive the progress of other breast cancers and present targets for hormonal treatments or HER2-targeted therapies. Because these targets are absent, the treatment options for TNBC are relatively limited and focused on chemotherapy, radiation, and newly established immunotherapies.[1,2]

TNBC is considered to be more aggressive and with a worse prognosis compared with hormone-receptor-positive breast cancer. It further occurs in more women who is young and African women or Hispanic origin.[3] Moreover, the metastatic potential of TNBC is higher especially towards visceral organs and the brain. For its unique biology, much interest has been taken about discovering novel biomarkers besides therapeutic targets that may lead to much better treatment and prognosis in patients with TNBC.[4,5]

Matrix Metalloproteinase-9 or MMP-9 is a family of proteases called matrix metalloproteinases which have established a critical role in degrading the extracellular matrix.[6]It has been implicated in significant biological processes, such as tissue remodeling, inflammation, and angiogenesis. In cancer, including TNBC, MMP-9 is of interest because it degrades basement membranes and ECM components to allow tumor cell invasion and metastasis.[6–8] Therefore breakdown of type IV collagen in the basement membrane by MMP-9 can facilitate the invasion of tumour cells into the surrounding tissues and may increase the chance of metastasis. MMP-9 can also release vascular endothelial growth factor (VEGF) from the ECM, thus contributing to angiogenesis - very important to tumor growth and metastasis.[8,9] High expression of MMP-9 has been seen in aggressive TNBC phenotypes and poor survival outcomes.8 Immunohistochemistry studies have shown a direct relationship of high levels of MMP-9 with poor differentiation, high-grade tumors, and shorter overall survival; this makes MMP-9 a potential marker in TNBC prognosis.[9]

Interleukin-8 (IL-8) is an important pro-inflammatory cytokine that is involved in inflammation and progression associated with cancer and metastasis.[10] IL-8 is a member of the CXC chemokines, which have the hallmark function of neutrophil chemotaxis and plays an important role in angiogenesis, particularly in the milieu of the tumor microenvironment.[11] IL-8 has its effects through its two receptors CXCR1 and CXCR2 in cancer cells promoting their survival, proliferation, and migration. It can also bring about the epithelial-mesenchymal transition (EMT), which is an important process in the metastasis of cancer cells. IL-8 is a potent pro-angiogenic factor, inducing new vessel formation, very essential for the growth of a tumor.[12] It also promotes metastasis by accelerating the motility of cancer cells. The proinflammatory setting can be provided by IL-8, recruiting neutrophils and macrophages, both of which can paradoxically promote tumor growth.[13] Such overexpression of MMP-9 and IL-8 has been linked to a more aggressive nature of TNBC and worse prognosis. Overexpression of IL-8 can be correlated

with increased metastasis and shorter survival, along with chemotherapy resistance.[14]

2 Methodology

A cross-sectional study was carried out at the NABL-accredited laboratory in the Department of Pathology, JSS Hospital, Mysuru, between January 2020 and May 2024. The study involved 29 paraffin blocks from patients who had undergone mastectomy or core biopsy following a triple-negative breast carcinoma diagnosis.

Inclusion criteria: Participants who are more than 18 years of age without any comorbidities and can provide the history and written consent.

Data collection tools: Data were extracted by using the backbone software used by JSS Hospital, and by asking the questionnaires based on the basic lifestyle and other required details which support the studies.

Staining technique: IHC is perhaps one of the most extensively used techniques to visualize specific expression of proteins in tissue samples-it provides very significant information regarding the tumor microenvironment, biology, and, therefore, future prognostic factors. Hence, IHC is frequently used in studies regarding MMP-9 and IL-8 expression in TNBC tumor and its stromal tissues. Utilizing a staining technique involving antibodies against MMP-9 and IL-8 will allow the determination of the presence and extent of these markers within the tumor microenvironment. The staining pattern often highlights MMP-9 in cancer cells, tumor-associated stromal cells, and areas of ECM degradation. High MMP-9 expression, particularly in the invasive front of the tumor or in peritumoral stromal cells, correlates with a more aggressive disease course. Quantifying MMP-9 levels can provide insights into the metastatic potential of TNBC. Elevated IL-8 staining is associated with increased tumor vascularization, inflammatory cell infiltration, and a higher metastatic risk. IHC results for IL-8 can help determine the tumor's angiogenic and inflammatory status, providing clues about its aggressiveness and potential therapeutic targets. H and E stain is used to stain the section to study clinicopathological parameters such as Lymphovascular invasion, perineural invasion, tumor budding, Km scoring. Grossing and macroscopical analysis were performed by using standard operating procedure. Figure 2 shows the expression of MMP-9 and IL-8 after IHC staining according to the standard reporting methods.

The Hematoxylin & Eosin (H&E) staining process starts with deparaffinization using xylene to remove paraffin, followed by fixation. The slides are then dehydrated through a graded alcohol series and rinsed with distilled water. Hematoxylin is applied to stain cell nuclei, and a bluing agent, such as lithium carbonate or tap water, is used to enhance nuclear staining. If necessary, differentiation with acid alcohol is performed. The slides undergo further dehydration before eosin is added to stain the cytoplasm. Another round of dehydration ensures proper stain retention, followed by clearing with xylene for better visualization. Finally, the slides are mounted with a coverslip and a suitable medium.

The Immunohistochemistry (IHC) staining procedure begins with overnight incubation, followed by deparaffinization and fixation using xylene. The slides are then dehydrated with a graded alcohol series and distilled water. Antigen retrieval is carried out using either heat-induced or enzymatic methods, followed by two buffer washes. To minimize non-specific staining, a blocking step is included, followed by additional buffer washes. The primary antibody is applied and incubated for an hour before the slides are washed again. The target antibody is then added for 15 minutes, followed by another washing step. Next, a secondary antibody conjugated with HRP is applied and incubated for 30 minutes, with subsequent buffer washes. DAB chromogen is then introduced for five minutes to visualize staining, followed by further washes. Counterstaining is done using running tap water to enhance contrast. After dehydration through graded alcohol, the slides are mounted with a coverslip to complete the process.

2.1 Assessment of the IHC Staining: Assessment of MMP-9 and IL-8

This method of analyzing the immunoreaction involves both qualitative and quantitative assessments of staining intensity in the cytoplasm of neoplastic cells. Here's a breakdown of the scoring system used for qualitative estimation shown in Figure 2.

Intensity of stain	Reporting	
Absence of staining	Negative	Score 0
Slightly positive staining	1+	Score 1
Moderately positive staining	2+	Score 2
Strongly positive staining	3+	Score 3

2.2 Statistical Analysis

All analyses were conducted using MedCalc Software Ltd 2024. One-way Chi-square tests were utilized to assess the correlation among all parameters and protein expression measured by the immunohistochemistry (IHC) method in this study. Additionally, Fisher's exact test and two-way Chi-square tests were employed to examine the relationships between clinicopathological parameters and the biomarkers. Hazard ratios (HRs) along with 95% confidence intervals and a 5% margin of error were calculated. A p-value of less than 0.05 was deemed statistically significant for all statistical evaluations.

Table 1. Correlation of MMP-9 and IL-8 with clinicopathological parameters in triple-negative breast cancer patients (n=29)

Category	Parameters	MMP-9 (29)		P Value	IL-8 (29)		P Value
		Positive (9)	Negative (20)		Positive (20)	Negative (9)	
Age	≤50 years	5	10	0.7855	8	7	0.0642
	>50 years	4	10		12	2	
Size	≤2cm	3	2	0.1305	10	0	**0.01***
	>2cm	6	18		10	9	
Site	Left	6	17	0.2679	15	8	0.4013
	Right	3	3		5	1	
Lymphnode involvement	Metastasis	4	4	0.1806	5	2	0.8737
	No Metastasis	5	16		15	7	
Lymphovascular invasion	Identified	5	10	0.7855	10	5	0.7855
	Not identified	4	10		10	4	
Perineural invasion	Identified	0	2	0.334	1	1	0.5549
	Not identified	9	18		19	8	
Grade	I	—	—	—	—	—	—
	II	2	2	0.6317	1	1	0.608
	III	7	12		13	6	
Tumor budding	>10	4	13	0.3069	12	5	0.8252
	<10	5	7		8	4	
Km score	0	2	3	NA	6	4	NA
	1	3	11		9	5	
	2	1	1		2	0	
	3	3	0		3	0	
KI67	<11%	0	0	NA	0	0	NA
	11-30%	0	0		0	0	
	31-50%	1	2		1	2	
	51-70%	1	3		3	0	
	71-90%	5	15		13	6	
	>90%	0	0		0	0	
IL-8	Positive	8	4	**0.0006***	8	1	0.1263
	Negative	1	16		12	8	

Table 2. One-way Chi-square test is performed to see the correlation betweentriple-negative breast cancer and protein expression

Protein/ Biomarker	Parameters	Triple Negative (N=29)	Percentage (%)	P value (<0.05)	Degree of freedom
MMP-9	Positive	9	31.03448276	**0.0411***	1
	Negative	20	68.96551724		
IL-8	Positive	20	68.96551724	**0.0411***	1
	Negative	9	31.03448276		

Table 3. Fisher extraction test to find correlation between MMP-9 and IL-8 in triple-negative breast cancer patients (n=29)

Protein/ Biomarker	Positive	Negative	P value (<0.05)
MMP-9	9	20	**0.0042***
IL-8	20	9	

Table 4. One-way Chi-square test to see the correlation between each parameter in triple-negative breast cancer patients

Category	Parameters	MMP-9 (29)				IL-8 (29)			
		Positive (9)	1-way Chi-square-P	Negative (20)	1-way Chi-square-P	Positive (20)	1-way Chi-square-P	Negative (9)	1-way Chi-square-P
Age	≤50 years	5	0.73	10	1	8	0.3711	7	0.0956
	>50 years	4		10		12		2	
Size	≤2cm	3	0.31	2	**0.0003***	10	1	0	**0.0027***
	>2cm	6		18		10		9	
Site	Left	6	0.31	17	**0.0017***	15	**0.0253***	8	**0.0196***
	Right	3		3		5		1	
Lymphnode involvement	Metastasis	4	0.73	4	**0.0073***	5	**0.0253***	2	0.0956
	No Metastasis	5		16		15		7	
Lymphovascular invasion	Identified	5	0.73	10	1	10	1	5	0.7389
	Not identified	4		10		10		4	
Perineural invasion	Identified	0	**0.0027***	2	**0.0003***	1	**0.0001***	1	**0.0196***
	Not identified	9		18		19		8	
Grade	II	2	0.0956	2	**0.0075***	1	**0.0013***	1	0.0588
	III	7		12		13		6	
Tumor budding	>10	4	0.73	13	0.1797	12	0.3711	5	0.7389
	<10	5		7		8		4	
Km score	0	2	0.7477	3	**0.0002***	6	0.1116	4	**0.0265***
	1	3		11		9		5	
	2	1		1		2		0	
	3	3		0		3		0	
KI67	<11%	0	**0.0064***	0	**<0.0001***	0	**<0.0001***	0	**0.0005***
	11-30%	0		0		0		0	
	31-50%	1		2		1		2	
	51-70%	1		3		3		0	
	71-90%	5		15		13		6	
	>90%	0		0		0		0	

Table 5. One-way Chi-square test to see the correlation between eachMMP-9 and IL-8 in triple-negative breast cancer patients

Category	MMP-9 (29)				Category	IL-8 (29)			
	Positive (9)	1-way Chi-square-P	Negative (20)	1-way Chi-square-P		Positive (20)	1-way Chi-square-P	Negative (9)	1-way Chi-square-P
IL-8 Positive	8	**0.0196***	4	**0.0073***	MMP-9 Positive	8	0.3711	1	**0.0196***
IL-8 Negative	1		16		MMP-9 Negative	12		8	

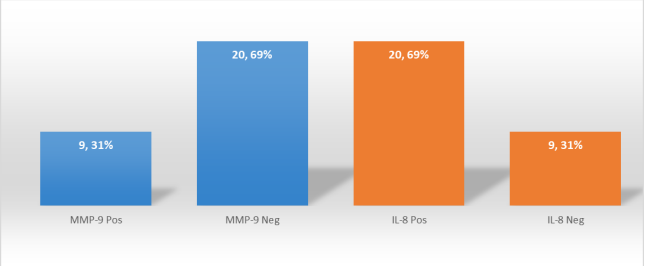

Fig 1. Graph showing the population of MMP-9 and IL-8 expression distribution in triple-negative breast cancer patients

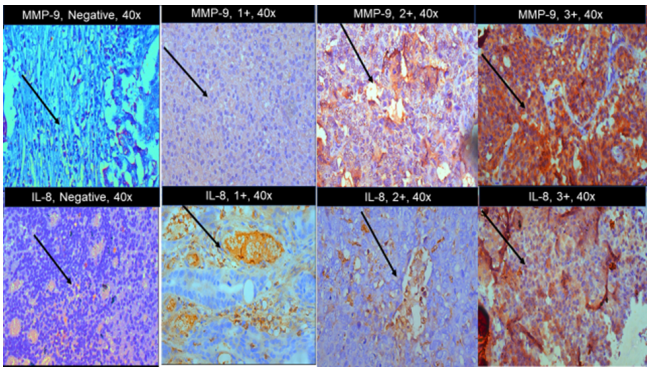

Fig 2. Showing the expression of MMP-9 and IL-8 in IHC staining

3 Results and Discussion

Correlation of MMP-9 and IL-8 with clinicopathological parameters in triple-negative breast cancer patients a total number of 29 cases were included in this study as shown in Figure 1, considering MMP-9 expression, in MMP-9 positive expression patients age ≤50 years are 17.24% and age >50 years were 13.79%, in tumor size ≤ 2cm were 10.34% and >2cm were 20.68%, considering the tumor site 20.68% of the cases were left and 10.34% of the cases were in right side, considering lymphnodes involvement 13.79% shows metastasis and 17.24% shows no metastasis, considering Lymphovascular invasion 17.24% of the patient who were identified and 13.79% were not identified, considering perineural invasion 31.03% were not identified, 6.89% of the patient falls under grade 2 and 24.13% of the cases falls under grade 3, 13.79% of the cases shows > 10 tumor budding and 17.24% of the cases shows <10 tumor budding, 6.89% of the cases falls under Km score 0, 10.34% falls under score 1, 3.44% of the cases falls under score 2, 10.34% of the cases falls under score 3, considering Ki 67 status majority of the cases were 17.24% which falls between 71-90% of cell proliferation rate, 27.58% of the cases shows IL-8 positive and 3.44% of the cases shows IL-8 negative as shown in the Table 4.

Considering MMP-9 negative expression patients age ≤50 years are 34.48% and age >50 years were 34.48% which shows similar cases, in tumor size ≤ 2cm were 6.89% and >2cm were 62.06% which was majority, considering the tumor site 58.62% of the cases were left and 10.34% of the cases were in right side, considering lymphnodes involvement 13.79% shows metastasis and 55.17% shows no metastasis, considering Lymphovascular invasion 34.48% of the patient who were identified and 34.48% were not identified, considering perineural invasion 62.06% were not identified, 6.89% of the patient falls under grade 2 and 41.37% of the cases falls under grade 3, 44.82% of the cases shows > 10 tumor budding and 24.13% of the cases shows <10 tumor budding, 10.34% of the cases falls under Km score 0, 37.93% falls under score 1, 3.44% of the cases falls under score 2, considering Ki 67 status majority of the cases were 51.72% which falls between 71-90% of cell proliferation rate, 13.79% cases were MMP-9 positive and 55.17% of the cases were MMP-9 negative which shows majority as shown in the Table 4.

Considering IL-8 positive expression patients age ≤50 years are 27.58% and age >50 years were 41.37%, in tumor size ≤ 2cm were 34.48% and >2cm were 34.48%, considering the tumor site 51.72% of the cases were left and 17.24% of the cases were in right side, considering lymphnodes involvement 17.24% shows metastasis and 51.72% shows no metastasis, considering Lymphovascular invasion 34.48% of the patient who were identified and 34.48% were not identified, considering perineural invasion 65.51% were not identified, 3.44% of the patient falls under grade 2 and 44.82% of the cases falls under grade 3, 41.37% of the cases shows > 10 tumor budding and 27.58% of the cases shows <10 tumor budding, 20.68% of the cases falls under Km score 0, 31.03% falls under score 1, 6.89% of the cases falls under score 2, 10.34% of the cases falls under score 3, considering Ki 67 status majority of the cases were 44.82% which falls between 71-90% of cell proliferation rate, 27.58% of the cases shows MMP-9 positive and 41.37% of the cases shows MMP-9 negative as shown in the Table 4.

Considering IL-8 negative expression patients age ≤50 years are 24.13% and age >50 years were 6.89% which shows similar cases, in tumor size ≤ 2cm were 0% and >2cm were 31.03% which was majority, considering the tumor site 27.58% of the cases were left and 3.44% of the cases were in right side, considering lymphnodes involvement 6.89% shows metastasis and 24.13% shows no metastasis, considering Lymphovascular invasion 17.24% of the patient who were identified and 13.79% were not identified, considering perineural invasion 27.58% were not identified and 3.44% were identified, 3.44% of the patient falls under grade 2 and 20.68% of the cases falls under grade 3, 17.24% of the cases shows > 10 tumor budding and 13.79% of the cases shows <10 tumor budding, 13.79% of the cases falls under Km score 17.24% falls under score 1, 0% of the cases falls under score 2, considering Ki 67 status majority of the cases were 20.68% which falls between 71-90% of cell proliferation rate, 3.44% cases were

MMP-9 positive and 27.58% of the cases were MMP-9 negative which shows majority as shown in the Table 4.

Considering the one-way Chi-square Test, we compare MMP-9 positive with clinicopathological parameters we found significance with perineural invasion, Ki 67, and IL-8 expression, considering MMP-9 negative expression we found significance with tumor size, locality, tumor grading, lymphnode involvement, perineural invasion, Ki 67, IL-8 status, Km score. And considering IL-8 positive expression with clinicopathological parameters we found significance with tumor site, lymphnode involvement, perineural invasion, grading, Km scoring, Ki 67. Also, considering IL-8 negative expression we found statistical significance with tumor size, locality of the tumor, perineural invasion, grading, Ki67 cell proliferation and MMP-9 expression as shown in Table 4.

Performing two-way Chi-square test we found that tumor size shows of statistical importance to IL-8 expression as shown in Table 1. A One-way Chi-square test is performed to see the correlation between triple-negative breast cancer and protein (MMP-9 and IL-8) expression which shows statistical significance as shown in Table 2. Also, by performing the Fisher extraction test to find the correlation between MMP-9 and IL-8 in triple-negative breast cancer patients it shows statistical significance as shown in Table 3.

4 Conclusion

Both MMP-9 and IL-8 play crucial roles in the pathophysiology of triple-negative breast cancer (TNBC) by promoting tumor invasion, metastasis, and a pro-tumorigenic microenvironment. Their detection via immunohistochemistry (IHC) provides valuable insights into TNBC biology and may aid in the development of targeted therapies. Our study suggests that high MMP-9 and IL-8 expression correlates with a more aggressive breast cancer phenotype, potentially increasing the risk of recurrence and reducing patient survival rates. These findings highlight the therapeutic potential of targeting MMP-9 and IL-8 through specific inhibitors or novel treatment approaches. However, further research is necessary to confirm these associations. Real-time molecular studies, advanced genetic analyses, and innovative methodologies are needed to enhance treatment strategies and improve patient outcomes.

5 Ethics statement

The study protocol was reviewed and approved by the Research Ethics Committee, JSS AHER, Mysuru. Informed consent was obtained from all participants.

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