



RESEARCH ARTICLE

Circulating MMP-9 and CYR61 Levels in HER2-Positive and -Negative Breast Cancer Subtypes

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ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women globally and a leading cause of cancer-related deaths. In Azerbaijan, breast cancer cases have steadily increased, with significant numbers recorded between 2020 and 2023. The human epidermal growth factor receptor 2 (HER2) gene is central in breast cancer pathology, particularly in aggressive forms with HER2 overexpression. However, emerging evidence shows that HER2 mutations may also occur in HER2-negative cases, potentially impacting prognosis and therapeutic outcomes.

Aim: This study investigates HER2 gene expression and mutation status in HER2-negative breast cancer cases and analyzes circulating matrix proteins (MMP-7, MMP-9, CYR61) in different breast cancer subtypes.

Methods: A total of 74 women aged 30–71 years with confirmed BC were enrolled. Patients were categorized as HER2-positive (n=33), HER2-negative (n=33), and triple-negative (n=8). HER2 mutation testing was conducted using AmoyDx kits via real-time PCR. Serum levels of MMP-7, MMP-9, and CYR61 were measured and statistically analyzed.

Results: No HER2 mutations were found in HER2-negative patients, supporting prior research indicating the rarity of such mutations in these subtypes. MMP-9 was significantly reduced in all breast cancer groups ($p < 0.001$), while CYR61 levels were markedly elevated, with an inverse correlation observed between MMP-9 and CYR61 ($r = -0.314$, $p < 0.003$). **Conclusion:** HER2 mutations are uncommon in HER2-negative breast cancers. Serum MMP-9 and CYR61 can serve as valuable diagnostic markers across BC subtypes. The findings support further exploration into alternative molecular targets beyond HER2 for effective treatment strategies.

Keywords: breast cancer; breast cancer subtypes; Her2-negative breast cancer; Her2-positive breast cancer; Her2 gene mutation, circulating matrix proteins: MMP-7, MMP-9 and CYR61; non-invasive biomarkers

Introduction

According to the World Health Organization (WHO), oncological diseases have remained among the leading causes of morbidity and mortality worldwide over the past decade. Statistical data demonstrate that breast cancer (BC) has shown the highest increase in incidence rates in recent years, currently ranking as the second leading cause of cancer-related deaths among women globally.¹⁻³ In Azerbaijan, BC has become the most commonly diagnosed cancer among women, with a steadily increasing incidence. According to the State Statistical Committee of the Republic of Azerbaijan, the number of breast cancer cases rose from 1,960 in 2020 to 2,638 in 2021, with a slight decrease to 2,541 in 2023.^{4, 5} In total, 13,666 new malignant tumor cases were diagnosed for the first time in 2023, while the total number of registered cancer patients reached 69,435. Breast cancer alone accounted for 18% of all newly diagnosed cancer cases in 2023—compared to 16.9% in 2020, 19.3% in 2021, and 18.4% in 2022.⁵

The etiology of breast cancer involves multiple factors. Without early detection and appropriate treatment, the disease can progress aggressively, leading to metastasis and, ultimately, fatal outcomes

The HER-2 (Human Epidermal Growth Factor Receptor 2) gene plays a crucial role in the development and progression of breast cancer.⁵⁻⁹ The *erbB2* gene, found on the short arm of chromosome 17, was cloned in 1983.²⁴ It encodes HER2, a transmembrane glycoprotein weighing 185 kDa, with three functional domains: extracellular, transmembrane, and intracellular, the latter possessing tyrosine kinase activity. HER2 is part of the epidermal growth factor receptor (EGFR) family, which includes four receptor tyrosine kinases.²⁵ Typically, HER-2 gene mutations are associated with HER-2 positive breast cancer, which is characterized by the overexpression or amplification of the HER-2 protein, leading to aggressive tumor growth.^{8,9} However, our research has begun to focus on HER-2 mutations in HER-2 negative breast cancer, where the HER-2 gene does not show amplification, but mutations still occur, potentially influencing cancer behavior and treatment responses. Studying HER-2 mutations in HER-2 negative breast cancer is an emerging and crucial area of cancer research, offering potential for more personalized treatment approaches, further understanding of the molecular mechanisms behind cancer development. This issue is particularly timely due to advancements in genomics and precision medicine, which are transforming cancer treatment paradigms.

Matrix metalloproteinases (MMPs), also known as matrixins, are proteolytic enzymes that remodel the extracellular matrix. They are involved in physiological processes such as morphogenesis, tissue regeneration, and reproduction, as well as in pathologies like arthritis, cancer, and cardiovascular disease. Moreover, matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that degrade various components of the extracellular matrix (ECM). MMPs are crucial in tumor invasion and metastasis by facilitating the breakdown of basement membranes and ECM, thus

enabling cancer cell migration. Among them, MMP-2 and MMP-9 are particularly implicated in breast cancer metastasis.^{10, 11}

Importantly, numerous studies have revealed a functional link between HER2 expression and MMP activity. HER2 overexpression in breast cancer tissues has been shown to upregulate the expression of MMP-9 through the activation of MAPK and PI3K/Akt pathways, thereby promoting tumor cell invasion and metastasis.¹²⁻¹⁴ Additionally, HER2 signaling may lead to increased secretion of MMP-2 and MMP-14, further enhancing ECM remodeling and angiogenesis.¹⁵

Emerging evidence suggests that this upregulation is not only restricted to tumor tissues but is also reflected in circulating levels of MMPs in the blood of breast cancer patients. Studies have reported elevated serum or plasma concentrations of MMP-2 and MMP-9 in HER2-positive patients compared to HER2-negative counterparts, indicating a systemic spillover effect potentially mediated by tumor-derived exosomes or cytokine signaling.^{16, 17} These circulating MMPs may serve as non-invasive surrogate biomarkers for tumor aggressiveness and HER2-driven activity.

Another molecule of interest is CYR61 (Cysteine-rich angiogenic inducer 61), an extracellular matrix-associated protein known to regulate cell adhesion, migration, proliferation, and apoptosis. Studies suggest that CYR61 may act downstream of HER2 signaling and synergize with MMPs to enhance tumor progression.¹⁸ The combined dysregulation of HER2 and matrix-related proteins like MMPs and CYR61 may represent a cooperative oncogenic network that significantly contributes to breast cancer invasiveness and poor prognosis. These findings underscore the importance of investigating HER2 expression in tissue alongside circulating matrix protein levels, particularly MMPs and CYR61, to better understand their molecular interplay and clinical significance in breast cancer pathogenesis.

The goal of the study is to investigate the expression levels of the HER2 gene in breast cancer tissues and the alterations of matrix metalloproteinases (MMPs) in patients' blood serum. By elucidating these molecular interactions, this study aims to contribute to a more comprehensive understanding of breast cancer biology and to identify potential targets for individualized therapy, particularly in HER2-negative subtypes where conventional HER2-targeted treatments are ineffective.

Patients, material and methods

Patients. The results of a prospective study conducted on 74 women aged 30-71 with BC who visited the National Oncology Center in 2023-2024 are presented. Immunohistochemical (IHC) analysis methods were used to examine the biotypes of breast cancer. Based on the results, 8 women were diagnosed with triple-negative phenotype, 33 women with HER-2 positive, and 33 women with HER-2 negative BC. Concentrations of MMP-7, MMP-9 and CYR-61 matrix-related proteins were measured in all enrolled breast cancer patients. For control was collected blood serum (n=15) in health pupils.

Sample Collection and Nucleic Acid Extraction. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks and peripheral blood samples were collected for molecular analysis. DNA and RNA were extracted from FFPE samples using the QIAamp DNA/RNA FFPE Tissue Kit (Qiagen, Cat. No. 56404). Genomic DNA was isolated from peripheral blood using the QIAamp Genomic DNA Extraction Kit (Qiagen, Cat. No. 56304).

HER2 Mutation Analysis. HER2 mutation testing was performed using the AmoyDx HER2 Mutation Detection Kit-24 (RFF: 8.01.0232). This assay is capable of detecting HER2 mutations in exon 20, including A775_G776insYVMA, M774_A775insAYVM, G776>V C, G776R, G776C, P780_Y781insGSP, and V777L, as well as exon 19 mutation L755P.

HER2 Gene Expression Analysis. In addition to mutation screening, HER2 gene expression levels were quantitatively assessed in FFPE tumor samples using SYBR Green-based real-time PCR and the ΔC_t method.²³ GAPDH was used as the endogenous control. The ΔC_t values were calculated by subtracting the C_t value of GAPDH from the C_t value of HER2. Lower ΔC_t values indicated higher HER2 expression. HER2-positive tumors demonstrated significantly lower ΔC_t values (mean $\Delta C_t = 4.2 \pm 1.1$) compared to HER2-negative tumors (mean $\Delta C_t = 9.6 \pm 1.3$), reflecting increased HER2 mRNA expression ($p < 0.001$, unpaired t-test). The primers for HER2 genes were designed based on their reference nucleotide sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI, USA) [<https://www.ncbi.nlm.nih.gov/genbank>]. Primer specificity was confirmed using the Primer-BLAST tool [<https://www.ncbi.nlm.nih.gov/tools/primer-blast>].

Primer sequences used:

HER2 Forward: 5'-ATATACCATGGCGATGCCCCGGGG GTCTCG-3'

HER2 Reverse: 5'-TTATACTCGAGCACTGGCACGTCCA GACC-3'

These molecular findings are consistent with the immunohistochemical HER2 classification and support the validity of using qPCR as a complementary diagnostic tool for HER2 expression profiling.

For biochemical analysis were collected 2 ml blood in tubes with anticoagulant (K3-EDTA). The samples were gently mixed to ensure proper. The concentrations of MMP-7, MMP-9 and CYR-61 in blood serum were determined using the Quantikine ELISA immunoassay method with reagent kits provided by Bio-Techne, R&D Systems (USA). The obtained results were measured using an automated immunoassay analyzer at a wavelength range of 450–630 nm.

Statistical Analysis

Statistical analyses were conducted using both parametric and non-parametric methods. Parametric tests included the independent samples t-test and Bonferroni correction, while the Wilcoxon–Mann–Whitney test was used for non-parametric comparisons. A p-value < 0.05 was considered statistically significant.

Results

Our results indicate an absence of detectable HER-2 mutations in patients with HER-2 negative breast cancer. MMP-7 levels in all BC subgroups were lower than in the control group but statistically significant differences were observed only using the Wilcoxon-Mann-Whitney test ($p < 0.05$). MMP-9 levels were significantly lower in BC patients compared to controls (53.3 vs. 486.9 ng/mL, $p < 0.001$). CYR61 was elevated in all cancer subtypes compared to controls: HER2-positive (406.6 ng/mL), HER2-negative (277.5 ng/mL), triple-negative (243.4 ng/mL) vs. control group (13.3 ng/mL).

Table 1. Serum level of MMPs and CYR-61 in patients with breast cancer

	MMP-7, ng/ml	MMP-9, ng/ml	CYR-61, pg/ml
Her2-positive, n=33	5,7 (0,2 – 32,2)	37,5 (8,0 – 140,0)	406,6 (0 – 5575,0)
Her2-negative, n=33	3,0 (0,2 – 25,4)	73,1 (8,0 – 688,0)	277,5 (0 – 784,0)
Triple-negative, n=8	5,7 (0,4 – 29,2)	37,0 (12,0 – 72,0)	243,4 (37,0 – 683,0)
Total BC, n=74	4,5 (0,2 – 32,2)	53,3 (8,0 – 688,0) *	331,4 (0 – 5575,0)*
Control, n=15	3,1 (1,8 – 4,1)	486,9 (269,0 – 884,0)	13,3 (0 – 149,0)

Note: * – Statistically significant difference compared to the control group at $p < 0.05$ level;

Matrix metalloproteinase 7 (MMP-7) levels were found to be slightly reduced in breast cancer (BC) patient groups compared to healthy controls. However, this reduction reached statistical significance only under non-parametric testing (Wilcoxon-Mann-Whitney test, $p < 0.05$), suggesting that while there may be a modest downregulation of MMP-7 in BC patients, the evidence is not sufficiently robust across all statistical methods. This indicates a potential but limited role for MMP-7 alterations in the pathophysiology of breast cancer that warrants further investigation.

In contrast, matrix metalloproteinase 9 (MMP-9) levels were significantly decreased in BC patients relative to

controls, with mean serum concentrations of 53.3 ng/mL and 486.9 ng/mL, respectively ($p < 0.001$). Given the well-established role of MMP-9 in extracellular matrix degradation and facilitation of tumor invasion and metastasis, this marked reduction in circulating MMP-9 may reflect complex tumor-host interactions or systemic regulatory mechanisms affecting MMP-9 expression or release. These findings highlight the importance of MMP-9 as a potential biomarker for breast cancer and suggest that altered serum levels could have diagnostic or prognostic relevance (figure 1).

Conversely, levels of CYR61, an extracellular matrix-associated protein involved in cell adhesion, migration,

proliferation, and apoptosis, were significantly elevated in all breast cancer subtypes analyzed. Specifically, mean serum CYR61 concentrations were 406.6 ng/mL in HER2-positive patients, 277.5 ng/mL in HER2-negative patients, and 243.4 ng/mL in those with triple-negative breast cancer, compared to 13.3 ng/mL in healthy controls ($p < 0.001$). This substantial increase underscores CYR61's potential role in breast cancer progression and its utility as a circulating biomarker indicative of tumor activity.

Importantly, a notable inverse correlation was identified between MMP-9 and CYR61 levels ($r = -0.314$, $p <$

0.003), suggesting a functional interplay between these two biomarkers. This negative association implies that as CYR61 levels rise, MMP-9 levels tend to decrease, highlighting a possible regulatory or compensatory mechanism within the tumor microenvironment. The reciprocal relationship between MMP-9 and CYR61 may have significant implications for understanding the molecular pathways involved in breast cancer progression and could inform the development of combined biomarker panels to improve diagnostic accuracy and therapeutic monitoring.

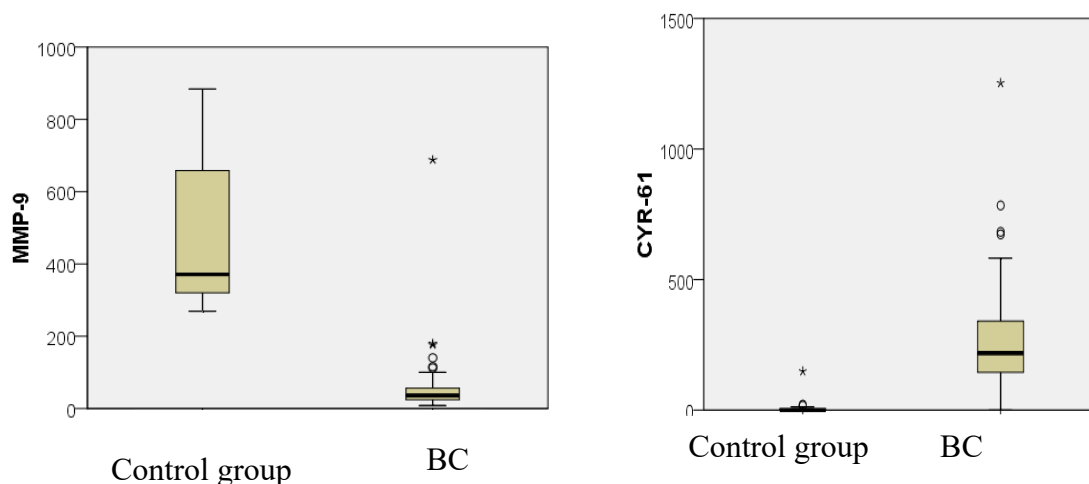


Figure 1. MMP-9 and CYR-61 level changes in breast cancer

Collectively, these results demonstrate significant alterations in serum levels of MMP-9 and CYR61 in breast cancer patients, reflecting their involvement in tumor biology and their potential clinical relevance as non-invasive biomarkers. Further studies are warranted to elucidate the mechanistic basis of their interaction and to validate their prognostic and therapeutic significance.

Despite no major differences among cancer subtypes, the difference between all BC patients and the control group was statistically significant for both MMP-9 and CYR61 ($p < 0.001$). A negative correlation was found between MMP-9 and CYR61 levels ($r = -0.314$, $p < 0.003$), suggesting an inverse relationship between these markers.

Discussion

Our findings, which show no detectable mutations in HER-2 in the HER-2 negative breast cancer patients, are consistent with several studies in the literature. For example, research by Huang et al. (2021), published in *Breast Cancer Research*, found that mutations in the HER-2 gene were rare in HER-2 negative breast cancers.¹⁹ Their study focused on patients with estrogen receptor-positive (ER+) and triple-negative breast cancers, revealing no significant HER-2 mutations in these groups. Similarly, Lo et al.²⁰ in *BMC Cancer* observed that only a small fraction (2%) of HER-2 negative, estrogen receptor-positive breast cancer samples showed slight amplifications of the EGFR gene, further suggesting the absence of significant HER-2 alterations in these subtypes.

Moreover, Wang et al. (2021), in their study published in *Journal of Clinical Oncology*, reported that in triple-negative breast cancers (TNBC), which often overlaps with HER-2 negative subtypes, EGFR mutations were more commonly found, but HER-2 mutations were not observed. This finding suggests that while EGFR mutations may play a role in some aggressive breast cancer cases, HER-2 mutations are not a frequent event in HER-2 negative breast cancers.²¹

Despite the potential implications of HER-2 mutations in treatment resistance, especially in relation to trastuzumab or lapatinib therapy, our study contributes to the growing body of evidence suggesting that mutations in HER-2 are not a major driver in HER-2 negative breast cancer. This contrasts with the commonly held view that HER-2 mutations could provide resistance to therapies targeting the HER-2 receptor. Instead, other pathways, such as mutations in the PIK3CA gene, EGFR amplifications, and PI3K-AKT-mTOR signaling pathways, may be more relevant in driving resistance in these cases, as highlighted by studies from Chung et al. (2018) in *Cancer Research*.²²

The lack of HER-2 mutations in our cohort also raises the question of alternative therapeutic strategies for HER-2 negative breast cancer. Since HER-2 mutations are not implicated in driving the malignancy in our study, future research should focus on identifying other molecular biomarkers that could predict therapeutic response and help guide treatment decisions. This includes examining the role of EGFR mutations, PI3K pathway alterations,

and mutations in tumor suppressor genes, all of which have shown to be more prominent in the absence of HER-2 amplification or overexpression. Our findings support the conclusion that HER-2 mutations are infrequent in HER-2 negative breast cancer, particularly in estrogen receptor-positive (ER+) and triple-negative (TNBC) subtypes. These results are consistent with prior research that has highlighted the limited role of HER-2 mutations in driving tumorigenesis in HER-2 negative breast cancer. Further research should focus on identifying other key mutations or alterations that may play a more significant

role in the progression of HER-2 negative breast cancer and resistance to targeted therapies.

Conclusion

Serum levels of MMP-9 and CYR61 are significantly altered in breast cancer patients and can serve as valuable diagnostic biomarkers regardless of tumor subtype. These findings emphasize the role of extracellular matrix-related proteins in the early detection of breast cancer and merit further exploration across other cancer types.

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