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Differential expression of metallothionein and p21 in gastric cancer and some precursor lesions

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Abstract. – OBJECTIVE: Gastric cancer (GC) is a heterogeneous disease with molecular diversity between and within tumors; therefore, searching for altered genes within this cancer is mandatory to reach the proper individualized targeted therapy. Expressions of Metallothionein (MT) and p21 are not uniform in various types of cancers and their predictive value in GC is controversial. This study aimed to assess the role of MT and p21 in intestinal-type GC and some of its precursor lesions.

MATERIALS AND METHODS: Immunohistochemical staining for MT and p21 was applied on paraffin blocks belonging to 30 GCs and 51 benign gastric lesions/precancerous lesions [33 chronic gastritis and 18 chronic gastritis with gastric intestinal metaplasia (GIM)]; 27 of them were associated with H. pylori infection.

RESULTS: MT expression was dramatically increased while p21 expression was dramatically decreased from chronic gastritis to GIM to GC. In precancerous lesions, H. pylori-positive cases had significantly higher MT expression and lower p21 expression compared to H. pylori-negative cases. In GCs, decreased expression of both MT and p21 was associated with high-grade and advanced-stage cancers.

CONCLUSIONS: Both MT and p21 may have a role in the development and progression of GC, and both proteins may be useful for selecting targeted therapy for GC patients.

Key Words:
MT, p21, H. pylori, Intestinal metaplasia, Gastric cancer.

Introduction

Gastric cancer (GC) is a major disease worldwide, ranking fifth in incidence and fourth in mortality globally[1]. Increased GC incidence in young adults (<50 years of age) is a recent demographic finding[2]. In Egypt, GC is the tenth most frequent cancer and the ninth cause of cancer-related deaths[3].

Helicobacter pylori (*H. pylori*) infection causes one of the most prevalent chronic bacterial infections in the world, with a prevalence of up to 50%[4]. *H. pylori* were classified as a type I carcinogen, since it has been related to the onset of chronic active gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and intestinal-type gastric adenocarcinoma[5].

Gastric intestinal metaplasia (GIM) is a precancerous lesion that affects nearly 25% of people globally. GIM might precede the onset of dysplasia[6]. More than 50% of patients with high-grade dysplasia will develop GC[7].

Metallothioneins (MTs) are a family of low molecular metal-binding proteins that play a role in a variety of pathological processes, including intracellular storage, transport, and metabolism of metal ions[8]. They also have a role in various carcinogenic processes as promoting tumor growth and inhibiting apoptosis[9].

The role and expression of MT differ in various kinds of cancers. MT expression was found to be elevated in cancers, such as cancers of breast, colon, kidney, lung, thyroid, and urinary bladder[10], while MT expression was downregulated in cancers like hepatocellular carcinoma[11]. The role of MT in GC remains unclear.

P21, a cyclin kinase inhibitor (CKI) from the Cip/Kip family, is a critical regulator of cell cycle progression during the G1 phase. It is involved in cell proliferation, terminal differentiation, stem cell phenotypes, apoptosis, and cellular stress response[12]. P21 aberrant expression was considered to play a key function in carcinogenesis progression[13]. Although p21 expression has been linked to a variety of cancers, the impact of p21 levels on GC disease development and prognosis is still controversial.

This study aims to assess the expression of MT and p21 in intestinal-type gastric cancer and its precursor lesions, such as chronic gastritis, *H. pylori* gastritis, and intestinal metaplasia.

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Materials and Methods

This retrospective study enrolled 81 cases with gastric lesions, which were divided into 30 cases who underwent subtotal or total gastrectomy with proven diagnosis of intestinal-type gastric cancer and 51 cases with benign gastric lesions/precancerous lesions (33 cases with chronic gastritis and 18 cases with GIM on top of chronic gastritis); 27 of which were associated with *H. pylori* infection. Cases were selected from the archive of the Pathology Department, Theodor Bilharz Research Institute (TBRI) from January 2019 to December 2020. The patients' demographic data were gathered from their medical records. Inclusion criteria included cases with chronic gastritis, *H. pylori* gastritis, GIM, intestinal-type GC, and GCs from the fundus/body area. Exclusion criteria included cases with autoimmune gastritis, cases who received *H. pylori* eradication therapy or received non-steroidal anti-inflammatory drugs, gastric malignancies other than intestinal-type GC, GCs at the cardia, and GCs previously treated with chemotherapy or radiotherapy.

The protocol of this study was reviewed and approved by the Institutional Review Board (IRB) of TBRI, for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland (2013). The IRB of TBRI waived the need for informed consent from the participants because the study was performed on stored archival tissue blocks. Personal information of these blocks' owners was anonymous and cannot reasonably be used by anyone.

Histopathological Technique and Evaluation

Paraffin-embedded sections were cut in a thickness of 4 μm, stained by hematoxylin and eosin (H&E) stain for routine histopathological examination and diagnosis. Sections were also stained by Giemsa stain for detection of *H. pylori*.

Immunohistochemical (IHC) Technique

One paraffin-embedded block was selected from each case and cut into 4 μm sections. The sections were put in the oven at 60°C for 4 hours, deparaffinized in xylene, and rehydrated in a graded ethanol series. Antigen retrieval was performed with 10 ml sodium citrate buffer, pH 6.0, at 90°C for 30 minutes. Sections were incubated in 0.03% hydrogen peroxide (EnVision/HRP, Dako) for 10 minutes at room temperature, then were rinsed in wash buffer.

The sections were incubated overnight at 4°C with MT at dilution of 1:100 (Biospes, Catalog No. YPA1550, Chongqing, China) and p21 at dilution of 1:25 (clone SX118, Dako, Denmark). Sections were washed three times for 5 minutes in PBS. Non-specific staining was blocked with 0.5% casein and 5% normal serum for 30 minutes at room temperature. Finally, staining was developed with diaminobenzidine substrate, then sections were counterstained with hematoxylin, dehydrated with graded ethanols, and mounted.

For each setting, positive and negative controls were routinely used. Positive controls were breast cancer for MT and skeletal muscle for p21. As a negative control, gastric tissue was processed, but the primary antibodies were not added, while non-immune immunoglobulin G (IgG; DAKO, Glostrup, Copenhagen, Denmark) was added.

Scoring of the Immunostained Tissue Sections

The immunostained tissue sections were independently reviewed and scored under a microscope by two pathologists. Brown color in the nucleus of the gastric cells was considered as positive staining. A total of 10 fields were randomly selected at high magnification (x400). MT and p21 staining were interpreted as positive when >10% of gastric cells showed distinct nuclear staining[14].

Statistical Analysis

Analyses were performed using SPSS version 23 (IBM Corp., Armonk, NY, USA). The significance of differences in means was calculated using *t*-test. Fischer's exact test was used to assess the significance of differences between clinicopathological variables. The correlations between the variables were assessed using the two-tailed Spearman correlation test. Differences were considered statistically significant whenever the *p*-values were <0.05.

Results

Patient Characteristics

This study enrolled a total of 81 benign (precancerous lesions) and malignant gastric lesions (Figure 1 a-d). Lesions belonged to 40 males (49.4%) and 41 females (50.6%), with a mean age of 47.28 ±14.51 years and 39.63 ±11.24 years respectively, with a significant difference in the mean age between both ($p<0.01$). There was a significant difference ($p<0.001$) between the me-

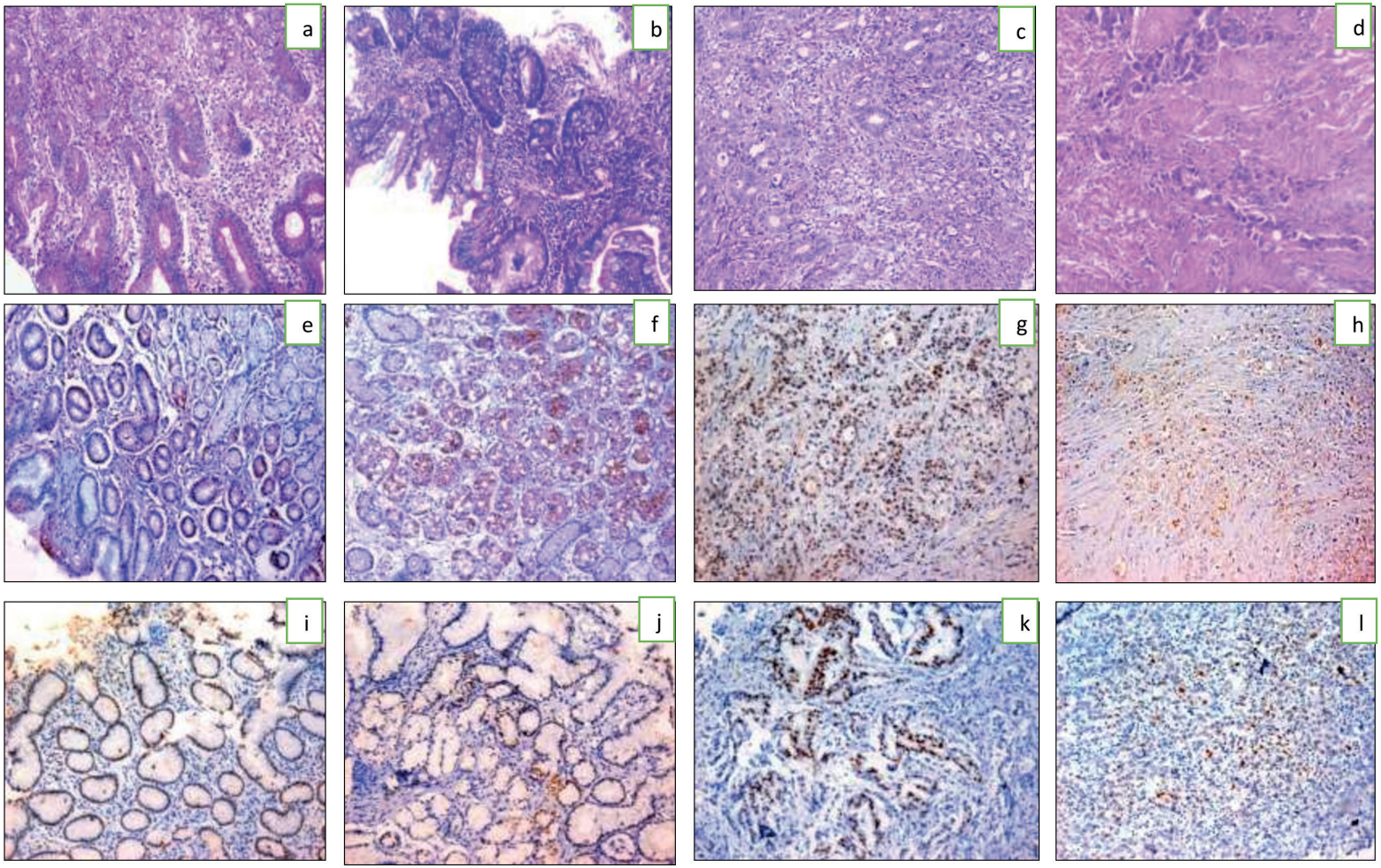

Figure 1. Hematoxylin & Eosin (Hx&E) staining of gastric lesions **(a-d)** and immunohistochemical (IHC) expression of metallothionein **(e-h)** and p21 **(i-l).** **(a)** Hx&E of chronic gastritis (×200), **(b)** Hx&E of chronic gastritis with intestinal metaplasia (×200), **(c)** H&E of low-grade gastric adenocarcinoma (×200), **(d)** Hx&E of high-grade gastric adenocarcinoma (×200), **(e)** metallothionein in chronic gastritis (X100), **(f)** metallothionein in chronic gastritis with intestinal metaplasia (×100), **(g)** metallothionein in low-grade gastric adenocarcinoma (×100), **(h)** metallothionein in high-grade gastric adenocarcinoma (×100), **(i)** p21 in chronic gastritis (×200), **(j)** p21 in chronic gastritis with intestinal metaplasia (×200), **(k)** p21 in low-grade gastric adenocarcinoma (×200), **(l)** p21 in high-grade gastric adenocarcinoma (×200).

Table I. Immunoreactivity of metallothionein (MT) and p21 in studied groups.

		MT immunoreactivity			P21 immunoreactivity		
Item (n.)		**Positive n. (%)**	**Negative n. (%)**	***p*-value**	**Positive n. (%)**	**Negative n. (%)**	***p*-value**
Benign gastric diseases (51)		**29 (57)**	**22 (43)**	<0.001*	**40 (78.4)**	**11 (21.6)**	<0.001*
Chronic gastritis (33)		15 (45.5)	18 (54.5)	0.025	27 (81.8)	6 (18.2)	0.325
GIM with chronic gastritis (18)		14 (77.8)	4 (22.2)		13 (72.2)	5 (27.8)	
***H. pylori* association in benign gastric diseases (51)**							
H. pylori-positive gastritis (27)		18 (66.7)	9 (33.3)	0.112	16 (59.3)	11 (40.7)	<0.001
H. pylori-negative gastritis (24)		11 (45.8)	13 (54.2)		24 (100)	0	
Gastric cancer (30)		**30 (100)**	**0**	<0.001** 0.016***	**12 (40)**	**18 (60)**	<0.001** 0.030***
Grade of differentiation	Low grade (18)	8 (100)	0		8 (44.4)	10 (55.6)	0.412
	High grade (12)	7 (100)	0		4 (33.3)	8 (66.7)	
Stage of invasion	Early stage (23)	23 (100)	0		10 (43.5)	13 (56.5)	0.403
	Advanced stage (7)	7 (100)	0		2 (28.6)	5 (71.4)	
Total		**59 (72.8)**	**22 (27.2)**		**52 (64.2)**	**29 (35.8)**	

Metallothionein, MT; n, number; %, percentage; GIM, gastric intestinal metaplasia; *Compared to GC; **Compared to chronic gastritis; ***Compared to GIM.

dian age of the cases with benign gastric lesions (35.53 ± 8.02 years) and cases with GC (56.80 ± 9.63 years).

MT and p21 Immunoreactivity in Benign Gastric Lesions (Precancerous Lesions) vs. Gastric Cancer

All GC cases were positive for MT compared to 57% of benign gastric lesions with a significant difference (p<0.001). In the benign gastric lesions group, MT-positivity was significantly higher in GIM cases compared to chronic gastritis cases (p=0.025) (Figure 1 e-h). Meanwhile, 40% of GC cases were positive for p21 compared to 78.4% of the benign gastric lesions with a significant difference (p<0.001). We found no significant difference in p21 positivity between chronic gastritis and GIM cases (p=0.325) (Figure 1 i-l) (Table I).

MT and p21 Expressions in Benign Gastric Lesions (Precancerous Lesions) vs. Gastric Cancer

Regarding MT expression (percentage of positive gastric cells), GC cases had a significantly higher MT expression compared to benign gastric lesions (p<0.001). In the latter group, there was no significant difference in MT expression between chronic gastritis and GIM cases (p=0.283). Meanwhile, p21 expression was significantly lower in GCs compared to benign gastric lesions (p<0.001). In the latter group, there was a significantly lower p21 expression in GIM compared to chronic gastritis cases (p=0.002) (Table II).

MT and p21 Immunoreactivity and Expressions in H. Pylori-Positive vs. H. Pylori-Negative Benign Gastric Lesions

By studying *H. pylori*-associated benign gastric lesions, *H. pylori*-positive cases were more frequently positive for MT (66.7%) than *H. pylori*-negative cases (45.8%), without significant difference (p=0.112). In addition, *H. pylori*-positive cases had a significantly greater MT expression than *H. pylori*-negative cases (p=0.007) (Table I). Meanwhile, all *H. pylori*-negative cases were positive for p21, compared to 59.3% of *H. pylori*-positive cases with a significant difference (p<0.001). Furthermore, *H. pylori*-negative cases showed a higher p21 expression than in *H. pylori*-positive cases with a significant difference (p<0.001) (Table II).

MT and p21 Immunoreactivity and Expressions in Gastric Cancer

All GCs showed positive immunostaining for MT. The expression of MT was significantly higher in low-grade and early-stage GCs compared to high-grade and advanced-stage cancers respectively ($p<0.001$). p21 positive immunostaining was more frequent in low-grade (44.4%) and early-stage GCs (43.5%) compared to high-grade (33.3%) and advanced-stage cancers (28.6%) respectively, without significant differences. Moreover, p21 expression was higher in low-grade and early-stage gastric cancers than in high-grade and advanced-stage cancers respectively, without significant differences (Tables I, II).

By Spearman correlation test, grade and stage of GC had a significant inverse correlation with MT expression yet an insignificant inverse correlation with p21 expression (Table III).

Correlation Between MT and p21 Expressions with Age and Sex

There was a positive significant correlation between age and MT expression, while an inverse significant correlation between age and p21 expression. Regarding sex, we observed an increase in MT and p21 expressions in association with females in benign gastric lesions, while an increase in their expressions with males in GC cases without significant differences (Table IV).

Relation Between MT and p21 Expressions

Coexpression of MT and p21 was seen in 9/33 (27.3%) of chronic gastritis cases, 9/18 (50%) of GIM, and 12/30 (40%) of GCs (Table V). A significant negative correlation was found between MT and P21 expressions (r=-0.624, p <0.001) (Table III).

Discussion

The stage is the most significant prognostic factor in GC; nevertheless, as GC is not a uniform disease, the prognosis differs among patients with the same stage. As a result, research on novel prognostic biomarkers is in progress. We analyzed the immunohistochemical expression of MT and p21 in benign and malignant gastric lesions.

Considering MT expression, we detected MT immunopositivity in 100% of GCs, 45.5% of chronic gastritis, and 77.8% of GIM. Similarly, Eber et al[15] revealed MT expression in areas of GIM and at least in 90% of their gastric cancer cases.

Table II. Expressions of metallothionein (MT) and p21 in studied groups.

		MT expression (% of MT positive gastric cells)		P21 expression (% of p21 positive gastric cells)	
Item (n.)		**Mean ± SD**	***p*-value**	**Mean ± SD**	***p*-value**
Benign gastric diseases (51)		**23.24 ± 22.13**	<0.001*	**55 ± 32.64**	<0.001*
Chronic gastritis (33)		20.00 ± 24.56	0.283	65.15 ± 32.22	0.002
GIM with CG (18)		27.78 ± 16.47		36.67 ± 24.97	
***H. pylori* association in benign gastric diseases (51)**					
H. pylori-positive gastritis (27)		30.93 ± 23.41	0.007	40.74 ± 36.58	<0.001
H. pylori-negative gastritis (24)		14.58 ± 17.25		71.25 ± 17	
Gastric cancer (30)		**82.33 ± 12.02**	<0.001**,***	**18 ± 23.22**	<0.001** 0.012***
Grade of differentiation	Low grade (18)	87.78 ± 4.61	0.412	22.22 ± 26.02	0.229
	High grade (12)	74.17 ± 15.05		14.17 ± 17.69	
Stage of invasion	Early stage (23)	88.26 ± 4.42	0.403	20.65 ± 24.69	0.264
	Advanced stage (7)	62.86 ± 7		9.29 ± 15.92	
Total	**45.12 ± 34.40**			**41.36 ± 34.44**	

Metallothionein, MT; n, number; %, percentage; GIM, gastric intestinal metaplasia; *Compared to GC; **Compared to chronic gastritis; ***Compared to GIM.

We observed an increase in MT expression from chronic gastritis to GIM to GCs. Higher MT expression in GC compared to benign lesions might indicate an oncogenic role for MT. In agreement with our results, Galizia et al[16] reported that MT expression was significantly higher in GC cells than in nontumoral cells. On the contrary, other studies reported a lower MT expression in GC specimens than normal mucosa[9,17].

We found a significantly higher MT expression in *H. pylori*-positive chronic gastritis cases compared to *H. pylori*-negative ones. This agrees with Shibuya et al[18] who found that increased MT protects the cells against DNA damage induced by *H. pylori* infection. It is known that *H. pylori* infection stimulates the generation of reactive oxygen species (ROS) through an inflammatory cell response and *H. pylori* itself[19]. According to Mitani et al[12], MT can reduce ROS produced by *H. pylori*, suggesting that MT may have a protective role against tissue damage. However, the decrease of MT expression does not prevent tissue damage in *H. pylori*-positive gastric mucosa, leading to more severe gastritis and this phenomenon may be attributed to gastric carcinogenesis.

We detected lower MT expression in high-grade and advanced-stage GCs compared with low-grade and early-stage cancers without significant difference. In agreement with our results, Tuccari et al[17] showed that MT immunostaining was significantly lower in advanced gastric carcinoma cases. However, Janssen et al[9] stated that MT overexpression correlated with a more malignant character of the tissues, whereas Eber et al[15] did not find an association between MT expression and the tumor stage or the grade of differentiation.

Regarding p21 expression, we found that p21 positive staining was significantly higher in benign gastric lesions compared to GC, with p21 positive staining identified in 40% of GCs and loss of its staining in 60% of cancers. Similarly, Michalaki et al[20] reported loss of p21 staining in 65% of intestinal-type GC, while Yeşillik et al[21] reported p21 loss in a greater percentage (90%).

We detected a significant decrease of p21 expression from chronic gastritis to GIM to GCs. Lower p21 expression in GCs, compared to benign lesions, may reflect the role of p21 as a cell cycle inhibitor, preventing the transition from G1 to S phase or from G2 to mitosis, following DNA damage caused by inflammation or metaplasia. In agreement with our results, Luo et al[22] reported a significantly lower p21 expression in the GCs than in precancerous lesions and chronic gastritis. In addition, Yeşillik et al[21] reported a reduction of p21 expression from GIM to GC. They hypothesized that low p21 expression contributes to the progression of gastric precancerous lesions and occurs early in gastric carcinogenesis. However, Craanen et al[23] did not find significant differences in p21 median product scores between chronic gastritis and GIM.

We found a significantly lower p21 expression in *H. pylori*-positive chronic gastritis cases compared to *H. pylori*-negative cases. This can be explained by the possible role of *H. pylori* infection in apopto-

Table III. Correlation between metallothionein (MT) and p21 expressions and clinicopathologic parameters.

Spearman's rho		MT expression	P21 expression
Age	Correlation Coefficient	0.627**	-.440**
	Sig. (2-tailed)	0.000	0.000
Male sex	Correlation Coefficient	-0.267*	0.227
	Sig. (2-tailed)	0.016	0.042
H. pylori association in benign gastric diseases	Correlation Coefficient	-0.395**	0.414**
	Sig. (2-tailed)	0.004	0.003
Grade of differentiation in GC	Correlation Coefficient	-0.420*	-0.240
	Sig. (2-tailed)	0.021	0.201
Stage of invasion in GC	Correlation Coefficient	-0.760**	-0.222
	Sig. (2-tailed)	0.000	0.239
MT expression	Correlation Coefficient	1.000	-0.624**
	Sig. (2-tailed)	.	0.000
P21 expression	Correlation Coefficient	-0.624**	1.000
	Sig. (2-tailed)	0.000	

Metallothionein, MT; GC, gastric cancer. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

Table IV. Relation between sex with metallothionein (MT) and p21 immunoreactivity.

		Immunopositivity			Expression (% of positive gastric cells)	
Protein	Groups (n.)	Positive n. (%)	Negative n. (%)	*p*-value	Mean ± SD	*p*-value
MT	**Benign gastric diseases (51)**					
	Male (18)	9 (50)	9 (50)	0.331	19.72 ± 22.32	0.408
	Female (33)	20 (60.6)	13 (39.4)		25.15 ± 22.13	
	GC (30)			**0.248***		
	Male (22)	**22 (100)**	**0**	-	82.502 ± 12.22	0.962
	Female (8)	8 (100)	0		58.48 ± 12.22	
P21	**Benign gastric diseases (51)**					
	Male (18)	14 (77.8)	4 (22.2)	0.559	48.89 ± 32.16	0.321
	Female (33)	26 (78.8)	7 (21.2)		58.48 ± 32.89	
	GC (30)			**0.467***		
	Male (22)	**11 (50)**	**11 (50)**	0.073	22.72 ± 24.29	0.063
	Female (8)	1 (12.5)	7 (87.5)		5.00 ± 14.14	

Metallothionein, MT; GC, gastric cancer. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

Table V. Relation between MT and P21 expressions in studied groups.

	Chronic gastritis n. (%)	GIM n. (%)	GC n. (%)	*p*-value
$MT^+/P21^+$	9 (27.3)	9 (50)	12 (40)	$p<0.001$
$MT^-/P21^-$	0	0	0	
$MT^+/P21^-$	6 (18.2)	5 (27.8)	18 (60)	
$MT^-/P21^+$	18 (54.5)	4 (22.2)	0	

GIM, gastric intestinal metaplasia; GC, gastric cancer; n, number; %, percentage; Metallothionein, MT.

sis resistance linked to p21 expression. Meanwhile, Saf et al[24] reported contrary results, and Petersson et al[25] found no association between p21 expression and *H. pylori* infection. These contradictory results may be attributed to differences in bacterial genetics, host genetics, duration of infection, and other associated environmental variables.

We detected lower p21 expression in high-grade and advanced-stage GCs compared to low-grade and early-stage cancers without significant difference. In agreement with our results, Luo et al[22] reported that a decrease of p21 expression was associated with tumor dedifferentiation and depth of tumor invasion in GCs. Our findings were also supported by previous studies[26,27] which document an association between the loss of p21 expression and poor prognosis in many solid tumors. However, Craanen et al[23] and Muller et al[28] found no significant correlation between p21 expression with grade or stage of GC. These ambiguous results regarding the association between p21 expression with the grade or stage can be explained by the dual role of p21, which can act as a tumor suppressor or as an oncogenic factor[29].

Age and sex are important risk factors for many cancers and can influence the choice of therapy. In our study, we found a significant association between MT and p21 expressions in GC cases and males; however, Galizia et al[16] and Altun et al[30] found no association between MT and p21 expressions and age or sex in their studies.

The current study presents some advantages. First, it investigated MT and p21 in gastric precancerous lesions and intestinal-type gastric cancer lesions, while most of the previous studies discussed only one of these lesions. Second, it evaluated the effect of *H. pylori* infection and GIM on the expression of MT and p21. However, the main limitation of our study was the relatively small number of studied cases.

Conclusions

In the present study, we observed a progressive increase in MT expression but a gradual decrease in p21 expression from chronic gastritis to GIM to gastric cancer. The decrease of MT and p21 expression in gastric tumors is an indication of advanced malignancy. These findings suggest that MT and p21 are involved in the development and progression of gastric cancer, and both proteins may be beneficial in the selection of targeted therapy for gastric cancer patients.

Conflicts of Interest

The authors declare no conflicts of interest.

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The authors funded this research personally.

Authors' Contributions

N.S.H, M.M. designing study, acquiring data, analyzing data, and writing manuscript. M.A.A acquiring data and drafting manuscript. M.M.M, T.A. designed and revised the manuscript. All authors revised and approved the final manuscript.

Data Availability Statement

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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