Original Research Article

Microsatellite Instability and P53 Aberrant Expression in Gastric Adenocarcinoma: Immunohistochemical Assessment and Correlation with Clinicopathological Characteristics

Abstract

Background: Gastric cancer (GC) is among the five most frequent cancer worldwide, following lung, breast, colorectal, and prostate cancer. It is the 12th most prevalent cancer in both sexes in Egypt, accounting for 1.6% of all malignancies. Distinct molecular subtypes of gastric adenocarcinoma (e.g., microsatellite instable subtype and P53 aberrant subtype) have been reported by different classification systems. However, the relation of these molecular subtypes to different clinicopathological features is still controversial.

Aim of the work:to utilize the immunohistochemical expression of DNA MMR proteins (MLH1 and MSH2) and p53 to detect microsatellite unstable and P53 aberrant molecular types in gastric adenocarcinoma. Moreover, to correlate immunohistochemically detected microsatellite unstable and P53 aberrant molecular types of gastric carcinoma with different clinicopathological characteristics.

Material & Methods: The immunohistochemical expression of MLH1, MSH2, and P53 proteins was evaluated in 70 cases of gastric adenocarcinoma. **Results**: Microsatellite status/Mismatch repair status showed a statistically significant relation with WHO classification, tumor differentiation, lymph node status and TNM staging. P53 aberrant type showed a statistically significant relation with tumor differentiation, depth of tumor invasion, lymph node status and TNM staging.

Conclusions & Recommendations: Microsatellite instable GC and P53 aberrant GC are two distinct molecular subtypes of gastric adenocarcinoma with distinct clinicopathological features and different prognostic outcome. Microsatellite instable tumors are associated with good prognostic parameters while P53 aberrant tumors are associated with poor prognostic parameters. Both subtypes could be detected using immunohistochemistry and could represent potential targets for future therapeutic agents.

Key Words: MSI, MMR deficiency, MLH1, MSH2, P53, gastric adenocarcinoma

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Introduction

Gastric cancer (GC) is among the five most frequent cancer [1]and the third most common causes for cancer-related fatalities worldwide [2]. Its prevalence is geographically variable and the majority of instances take place in developing nations and high-risk regions including Central and South America, Eastern Europe, and East Asian nations. [3].

GC is a varied and complicated disease caused by many interactions between genetic, environmental, and host variables. Despite attempts to enhance treatment methods over the last several decades, GC still has poor results [4].

Gastric cancer was traditionally classified based on morphology. The World Health Organization (WHO) categorized gastric adenocarcinoma into five major patterns in 2019: tubular, papillary, poorly cohesive, mucinous, and mixed adenocarcinomas, whereas the Lauren classification categorized gastric adenocarcinoma into (diffuse, intestinal, and mixed patterns)[5,6]. These morphology-based classification systems can't convey molecular heterogeneity nor guide the clinical practice for assessing the prognosis or anticipating the response of the therapy of GC patients. Therefore, identifying different subtypes of GC depending ongenetic and molecular characteristics is essential for selection of targeted therapy [7]

The most thorough molecular categorization of GC was published in 2014 by The Cancer Genome Atlas (TCGA) Research Network. They suggested a molecular categorization that subcategorized GC into four subtypes: tumors with microsatellite instability (MSI), tumors with chromosomal instability (CIN), tumors positive for the Epstein-Barr virus (EBV), and tumors with genomic stability (GS) cancers[3].The TP53 mutation rate was greatest in the CIN subtype [7], and P53 protein aberrations by immunohistochemistry (IHC) were subsequently utilized as a substitute for the CIN subtype[5].

Four molecular subtypes of GC were identified by The Asian Cancer Research Group (ACRG) in 2015: MSI, Microsatellite stable and epithelial to mesenchymal transition (MSS/EMT), MSS and TP53 active (MSS/TP53+), and MSS and TP53 inactive (MSS/TP53-) which showed the greatest rate of TP53 mutation[4].

Later on, other studies reported more subtypes based on both TCGA and ACRG classifications [7]and the WHO (2019) stated that IHC of mismatch repair (MMR) proteins, P53 and epithelial-

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cadherin (E-cadherin) in combination with in situ hybridization (ISH) of EBV could allow molecular subtyping of GC in routine pathology [6].

MMR is the mechanism that recognizes and fixes base-base mismatches and insertions and deletions of DNA (deoxyribonucleic acid) that are created during the replication and recombination processes. This process is crucial for preserving genomic stability. Therefore, MMR faults are linked to genome-wide instability and a steady buildup of mutations, particularly in areas of the basic repeated DNA sequences known as microsatellites, which lead to MSI. [8]. Microsatellite instability is a resultant of defective MMR genes [mainly mutL homolog 1 (MLH1) and mutS homolog 2 (MSH2)] [9]. Therefore, identification of MSI can be accomplished by IHC of MMR proteins [1].

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MMR-deficient cancers are presently seen as attractive targets for immunological checkpoint inhibition based on programmed cell death protein 1/programmed death-ligand 1 (PD1/PD-L1). This is because MMR-deficient cancers have PD-L1 gene amplification in a hypermutated phenotype with an increased tumor mutational burden[10].

The most frequently altered gene in instances of GC, found in 40% of patients, is the TP53 gene. The tumor suppressor protein P53 is essential for the halt of the cell cycle, metabolism, senescence, apoptosis, and DNA repair. Moreover, an important role of P53 is to maintain genomic stability so, it is called ‘the guardian of the genome’[11].

P53 is the major executor of cell response to DNA damage and is thought to serve as a molecular hub for the communication between stressors (such as reactive oxygen species [ROS], nutritional deprivation, hypoxia, etc.) and cellular biological responsesso, P53 protein is defined as a stress response protein[12].

The present work aimed to utilize the immunohistochemical expression of DNA MMR proteins (MLH1 and MSH2) and p53 to detect microsatellite instabilityand P53 aberrant molecular types in gastric adenocarcinoma. Moreover, to correlate immunohistochemically detected microsatellite instability and P53 aberrant molecular types of gastric carcinoma with different clinicopathological characteristics.

Material and method

This retrospective investigation included 70 instances primarily diagnosed as gastric adenocarcinoma in gastrectomy specimens (including 30 total gastrectomy specimens and 40 distal gastrectomy specimens), collected from the Pathology Department (Faculty of Medicine-Tanta University), Tanta Cancer Center and private laboratories during the period from June 2020 to January 2022. Prior to starting the study, the Tanta University Faculty of Medicine's Research Ethics Committee (REC) gave its approval. Approval code # 33827/5/20.

Clinicopathological data:

Clinicopathological data such as age, gender, tumor location, tumor size and distant metastasis were obtained from the cases' clinical sheets and pathology reports from the Pathology department (Tanta University), Tanta Cancer Center, and private laboratories files.

Histopathological evaluation:

Surgical samples were embedded in paraffin wax after being preserved in 10% neutral buffered formalin. Formalin-fixed paraffin-embedded block sections 3-5 µm thick were taken, and they were meticulously examined using standard H&E staining to confirm the histopathological diagnosis and assess various histopathological features like differentiation, lymph node status (N), depth of invasion (T), perineural invasion, and vascular invasion.

Cases of gastric adenocarcinoma were microscopically categorized as papillary, tubular, mucinous, poorly cohesive carcinoma (which includes signet ring cell carcinoma), and mixed adenocarcinomas, according to the World Health Organization (WHO) classification system, 5th edition, 2019 [6].

Tumor differentiation:

Tubular adenocarcinoma cases were subclassified based on histopathological grade from well to poorly differentiated by WHO criteria (2019). Well-differentiated tubular adenocarcinomas are predominantly made up of properly formed glands. In contrast, poorly differentiated tubular adenocarcinomas are made up of extremely irregular, ill formed glands and may have solid portions or individual cells, and moderately differentiated tumors show features that are "intermediate" between well and poorly differentiated tumors [6].

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Regarding tumor differentiation, gastric adenocarcinoma cases were classified into differentiated and undifferentiated tumors. Differentiated tumors include moderately and well differentiated tubular and papillary adenocarcinomas, whileundifferentiated tumors include poorly differentiated tubular adenocarcinomas, poorly cohesive carcinomas (including signet ring cell carcinomas), and others [13,14].

Staging of gastric adenocarcinoma cases:

The pathological staging of the examined gastric adenocarcinoma patients has been defined using the TNM staging strategy in accordance with the guidelines of the 8th edition of the AJCC, Cancer Staging Manual, 2017 [15,16]

Immunohistochemical staining:

Routine formalin fixed paraffin embedded (FFPE) sections, cut at 3 µm, were collected on positivecharged slides.Slides were transferred to the Autostainer Link 48 instrument.Both high and low pH EnVision™ FLEX Target Retrieval Solutions (Dako, Burlington) were employed.Immunostaining was done with Dako and Spring bioscience primary antibodies. These were primarily Dako FLEX Ready to-Use format [MLH1 (*Kit no. E17810. Spring bioscience, Pleasanton, CA, USA*) and MSH2 *(Kit no. E17790. Spring bioscience, Pleasanton, CA, USA*). rabbit polyclonal antibodies, and P53 (*Kit no. M7001. Dako, Glostrup, Denmark*) mouse monoclonal antibody].The DakoEnVision^{TM} FLEX Detection system was then used, despite the absence of linker antibodies, in accordance with the standard protocol: 10 minutes for the peroxidase blocking reagent, 20–30 minutes for the primary antibodies, 20 minutes for the detection system, and 10 minutes for the chromogen (diamnobenzidene, or DAB). At the conclusion of the staining process, the slides were flooded with distilled water, a counter stain with Mayer's hematoxylin was applied for one minute, and the slides were finally washed in tap water. The slides were covered by using Canada balsam.[17]

Interpretation of MLH1 and MSH2 immunostaining

Tumor cells with brownish nuclear staining was considered positive regardless cytoplasmic staining. When there was no trace of nuclear staining in any of the tumor cells, a tumor was deemed to be devoid of MLH1 or MSH2 expression (negative). As an internal positive control, peri-tumorous lymphocytes, stromal cells, and non-neoplastic epithelial cells were used [18]

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Detection of microsatellite status (MS status)/Mismatch Repair status (MMR status):
Categorization of gastric carcinoma cases according to MLH1 and MSH2 expression is illustrated in table 1.

Table 1: Summary of the immunohistochemical interpretation of MLH1&MSH2

Category	Expression
MSI/MMR-deficient	MLH1 Negative or MSH2 Negative or Both MLH1 &MSH2 Negative
MSS/MMR-proficient	MLH1 Positive and MSH2 Positive

Mismatch repair status (MMR status) was determined by the immunohistochemical analysis of MLH1 and MSH2 expression [19].Tumors negative for either MLH1 or MSH2 nuclear expression in all tumor cells were classified as MMR-deficient, whereas tumors with nuclear expression of both MLH1 and MSH2 were considered MMR-proficient[9]irrespective of the number or intensity of the stained tumor cell nuclei [20]

Microsatellite status (MS status): Gastric adenocarcinomas with brownish nuclear immunostaining of MLH1 protein (MLH1 positive) and MSH2 protein (MSH2 positive) in neoplastic cells were categorized as microsatellite stable tumors (MSS tumors/ tumors negative for MSI). Tumors showing complete loss of nuclear immunostaining for MLH1 protein (MLH1 negative) or MSH2 protein (MSH2 negative), in neoplastic cells, were categorized as MSI tumors[21].

Interpretation of P53 immunostaining:
Strong diffuse nuclear staining in more than 90 percent of tumor cells was the definition given for **aberrant expression of P53**, while the total lack of P53 expression in all tumor cells was the alternative; P53 expression was categorized as **wild type**[5]

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Statistical analysis of the data:

Using software from the statistical package for the social sciences (SPSS), version 20.0, the gathered data was tabulated, arranged, and statistically evaluated. Calculations were done to determine the range, mean, and standard deviation of the quantitative data. The number of observations and the percentage of distribution were computed for the qualitative data. The significance of categorical variables was examined using the chi-square test so that comparisons could be made between the various groups. When more than 20% of the cells had an anticipated count of less than 5, Fisher's Exact correction and Monte Carlo correction were used as methods for correcting the chi-square test. Significance was adopted at p-value <0.05.

Results

This retrospective study was carried out on 70 cases primarily diagnosed as gastric adenocarcinomas. Clinicopathological variables of the studied cases are listed in **table2.**The median age of the studied cases was 65 years ranging from 23-73 years withmale predilection (38/70;54.3%). Most of the studied cases werelocated in antrum and pylorus (n=44;62.8%). The majority of cases measured more than 5 cm in diameter (50/70;71.4%). Tubular and poorly cohesive histological types were the most frequent in the studied cases (26/70;37.1% each). Forty-six cases (65.7%) showed undifferentiated morphology. Almost half of the cases (38/70; 54.3%) were T3 and 22 cases (31.4%) were N1 whereas stage II was detected in 32 cases(45.7%). Ten cases (14.3%) had distant metastasis. Lymphovascular invasion was detected in 34 cases(48.6%) and perineural invasion in 19 cases (27.1%).

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Table 2: Classification of the studied gastric carcinoma cases according to different clinicopathological parameters

Clinicopathological variables	Number (%)
Age	
<60 years	29 (41.4)
≥60 years	41 (58.6)
Gender	
Male	38 (54.3)
Female	32 (45.7)
Tumor location	
Cardia and fundus	16 (22.9)
Body	10 (14.3)
Antrum and pylorus	44 (62.8)
Tumor size	
≤ 5 cm	20 (28.6)
> 5 cm	50 (71.4)
Histopathological types	
Tubular	26 (37.1)
Papillary	4 (5.7)
Poorly cohesive	26 (37.1)
Mucinous	10 (14.4)
Mixed	4 (5.7)
Tumor differentiation	
Differentiated	24 (34.3)
Undifferentiated	46 (65.7)
Depth of invasion	
T1	4 (5.7)
T2	20 (28.6)

T3	38 (54.3)
T4	8 (11.4)
Lymph node status	
N0	18 (25.7)
N1	22 (31.4)
N2	20 (28.6)
N3	10 (14.3)
Distant metastasis	
M0	60 (85.7)
M1	10 (14.3)
TNM stage	
Stage I	6 (8.6)
Stage II	32 (45.7)
Stage III	22 (31.4)
Stage IV	10 (14.3)
Vascular invasion	
Present	34 (48.6)
Absent	36 (51.4)
Perineural invasion	
Present	19 (27.1)
Absent	51 (72.9)
Total	70 (100)

Immunohistochemical results

MLH1 & MSH2 immunostaining results and classification of the studied cases according to microsatellite (MS) status/ mismatch repair (MMR) status

Out of 70 gastric adenocarcinoma cases, 55 cases (78.6%) were classified as microsatellite stable (MSS)/ MMR-proficient cases showing combined positive nuclear immunostaining of both MLH1 and MSH2 **[Figure 1 and 2]** while the remaining 15 cases (21.4%) were classified as tumors with microsatellite instability (MSI)/mismatch repair deficient (MMR-deficient) cases including 9 cases showing only MLH1 negativity (complete loss of MLH1 nuclear immunostaining) with positive MSH2 nuclear immunostaining **[Figure 3 and 4]**and 6 cases showing combined MLH1 and MSH2 negativity**(**complete loss of MLH1& MSH2 nuclear immunostaining) **[Figure 5 and 6]** while none of the studied cases was only negative for MSH2 with positive MLH1 immunostaining.Distribution of the studied cases according to microsatellite (MS) status/mismatch repair (MMR) status is summarized in**table 3.**

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Table 3: Distribution of the studied gastric carcinoma cases according to microsatellite (MS) status/mismatch repair (MMR) status:

Microsatellite (MS) status/mismatch repair (MMR) status		Number (%)	
MSI/MMR-deficient	MLH1 negative (with positive MSH2)	15 (21.4)	9 (12.8)
	Both MLH1&MSH2 negative		6 (8.6)
MSS/MMR-proficient	Both MLH1&MSH2 positive	55 (78.6)	
Total		70 (100)	

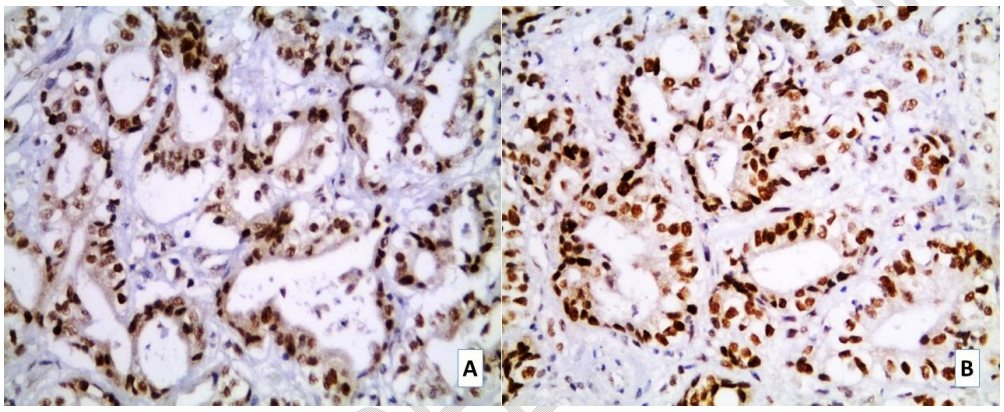

Figure 1: A case of moderately differentiated tubular adenocarcinoma classified as **microsatellite stable (MSS)/ MMR-proficient** showing combined nuclear positivity for **A**: MLH1 **B**: MSH2 immunostaining (*streptavidin biotin x400*).

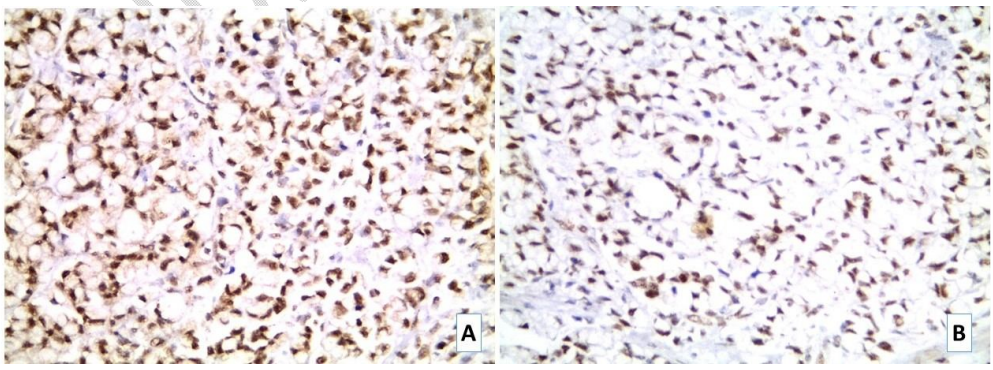

Figure 2:A case of poorly cohesive carcinoma, signet ring type adenocarcinoma classified as **microsatellite stable (MSS)/ MMR-proficient** showing combined nuclear positivity for **A**: MLH1 **B**: MSH2 immunostaining (*streptavidin biotin x400*).

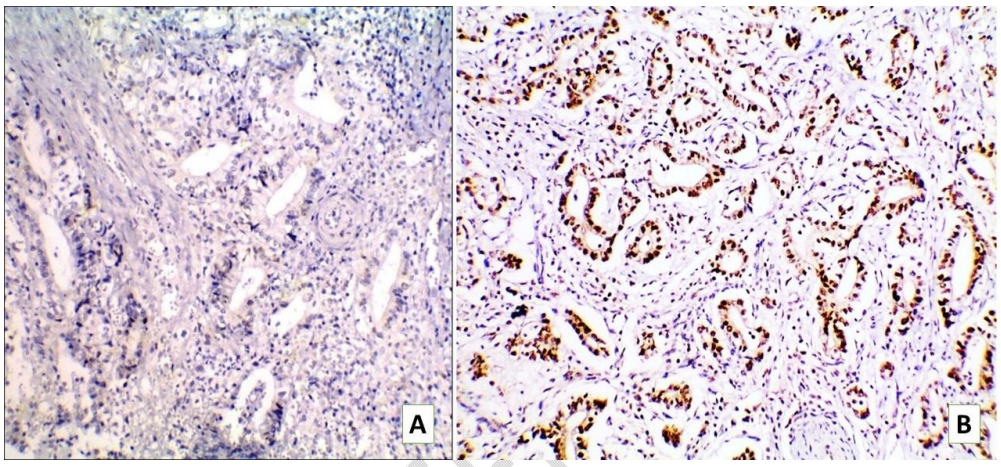

Figure 3: A case of well differentiated tubular adenocarcinoma**(MSI/MMR-deficient case**) showing **A**: negative MLH1 and **B**: positive MSH2 immunostaining (*streptavidin biotin x200*).

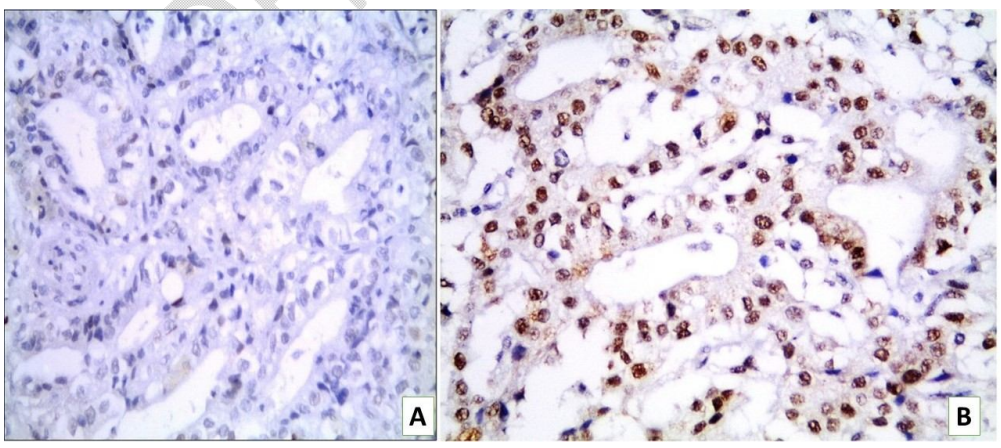

Figure 4:A case of moderately differentiated tubular adenocarcinoma **(MSI/MMR-deficient case**) showing **A:** negative MLH1 and **B:** positive MSH2 immunostaining (*streptavidin biotin x400).*

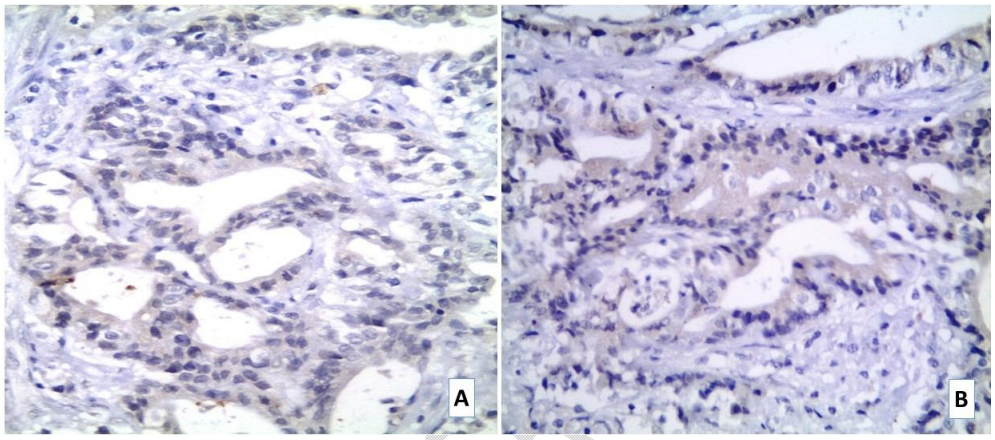

Figure 5: A case of moderately differentiated tubular adenocarcinoma classified as **MSI/MMR-deficient case** showing negative nuclear **A**: MLH1 and B: MSH2 immunostaining(*streptavidin biotin x400).*

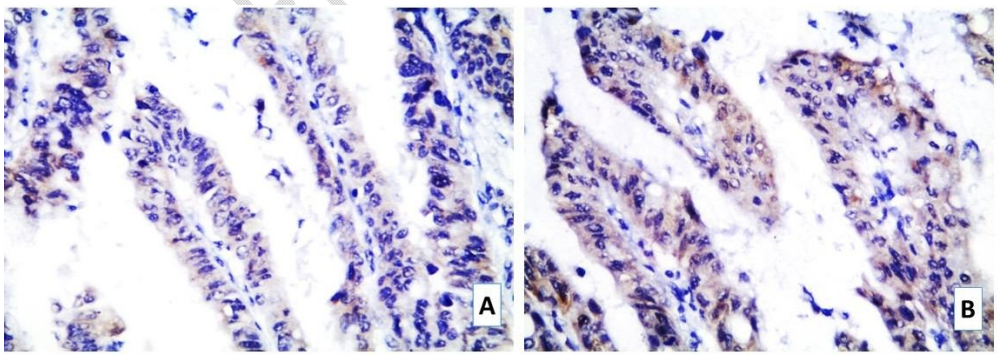

Figure 6:A case of papillary adenocarcinoma classified **as MSI/MMR-deficient case** showing negative nuclear **A**: MLH1 and **B**: MSH2 immunostaining (*streptavidin biotin x400*).

Relation between MS status/MMR status and clinicopathological characteristics of the studied cases of gastric carcinoma:

Relation between MS status/MMR status and the clinicopathological variables of the studied GC cases is listed in **table 4**. The relation between MS status/MMR status and histopathological types of the studied cases was statistically significant(with all papillary adenocarcinoma cases (100%) showing MMR deficiency/MSI) (p value=0.003). Regarding tumor differentiation, the relation between MS status/MMR status and tumor differentiation of gastric carcinoma cases was statistically significant with the majority of cases with MSI (9/15;60%) were of differentiated type (p value =0.018).

MS status/MMR status and lymph node status (N) was statistically significant with MSI/MMR-deficient cases associated withlower rate of lymph node metastasis (N0=7/15;47% and N1=6/15;40%) (LowerpN stage) compared to MSS/MMR-proficient cases (p value=0.045). The relation between MS status/MMR status and TNM staging of the studied cases was statistically significant with most cases with MSI were of stage I & II (13/15;87%) (Early stage) (P value=0.022).

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Table 4: Relation between MS status/MMR status and the clinicopathological variables of the studied gastric carcinoma cases

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Clinicopathologic variables	Total	MS status/MMR status		χ^2	P
		MSI/MMR-deficient	MSS/MMR-proficient		
		N. (%)	N.(%)		
Age					
<60 years	29	4 (13.8)	25 (86.2)	1.714	0.190
≥60 years	41	11 (26.8)	30 (73.2)		
Gender					
Male	38	6 (15.8)	32 (84.2)	1.570	0.210
Female	32	9 (28.1)	23 (71.9)		
Tumor location					
Cardia and fundus	16	3 (18.8)	13 (81.2)		

Body	10	2 (20.0)	8 (80.0)	0.124	1.000
Antrum and pylorus	44	10 (22.7)	34 (77.3)		
Tumor size					
≤ 5 cm	20	3 (15.0)	17 (85.0)		
>5 cm	50	12 (24.0)	38 (76.0)	0.687	0.528
Histopathological type					
Tubular	26	5 (19.2)	21 (80.8)		
Papillary	4	4 (100)	0 (0.0)		
Poorly cohesive	26	4 (15.4)	22 (84.6)		
Mucinous	10	2 (20)	8 (80.0)	16.40	0.003*
Mixed	4	0 (0.0)	4 (100.0)		
Tumor differentiation					
Differentiated	24	9 (37.5)	15 (62.5)		
Undifferentiated	46	6 (13.0)	40 (87.0)	5.603	0.018*
Depth of tumor invasion					
T1	4	1 (25)	3 (75.0)		
T2	20	6 (30)	14 (70.0)		
T3	38	8 (21.1)	30 (78.9)	3.088	0.371
T4	8	0 (0.0)	8 (100.0)		
Lymph node extension					
N0	18	7 (38.9)	11 (61.1)		
N1	22	6 (27.3)	16 (72.7)		
N2	20	2 (10.0)	18 (90.0)	7.984	0.045*
N3	10	0 (0.0)	10 (100.0)		
Tumor stage					
Stage I	6	3 (50)	3 (50.0)		
Stage II	32	10 (31.2)	22 (68.8)		
Stage III	22	2 (9.1)	20 (90.9)	9.459	0.022*
Stage IV	10	0 (0.0)	10 (100.0)		
Vascular invasion					
Absent	36	9 (25.0)	27 (75.0)		
Present	34	6 (17.6)	28 (82.4)	0.561	0.454

Perineural invasion					
Absent	51	10 (19.6)	41 (80.4)		
Present	19	5 (26.3)	14 (73.7)	0.370	0.531

***P value less than 0.5 was considered statistically significant**

P53 immunohistochemical results and classification of the studied gastric adenocarcinoma cases according to P53 expression into aberrant and wild types

Representative images of p53 immunostaining are demonstrated in **[Figure 7 and 8]**Out of 70 gastric adenocarcinoma cases, 36 cases (51.4% of the studied cases) were classified as P53 aberrant type including 29 cases (41.4% of total cases) showing strong diffuse nuclear P53 immunostaining in more than 90% of tumor cells and 7 cases (10.0% of total cases) showing complete absence of P53 expression in all tumor cells. Thirty-four cases (48.6% of the studied cases) were classified as P53 wild type.Classification of the studied gastric carcinoma cases according to the P53 type is listed in **Table 5.**

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Table 5: Classification of the studied gastric carcinoma cases according to P53 expression:

	P53 type	N. (%)	
P53 aberrant type	Strong diffuse immunostaining	36 (51.4)	29 (41.4)
	Negative immunostaining		7 (10.0)
P53 wild type		34 (48.6)	
	Total	70 (100.0)	

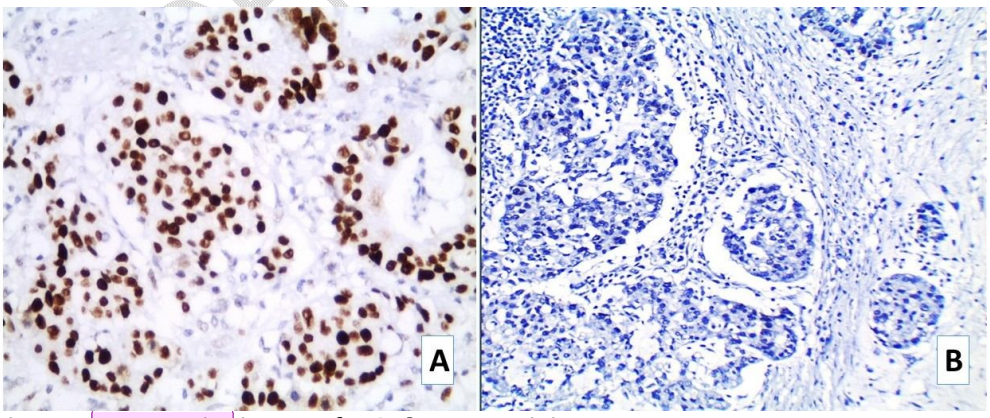

Figure 7:Representive images of p53 aberrant staining patterns:

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A: case of poorly differentiated tubular adenocarcinoma, P53 aberrant type (***strong diffuse nuclear*** P53 immunostaining) (*streptavidin biotin x400*).

B: A case of poorly differentiated tubular adenocarcinoma, P53 aberrant type (***negative nuclear*** P53 immunostaining) (*streptavidin biotin x200*).

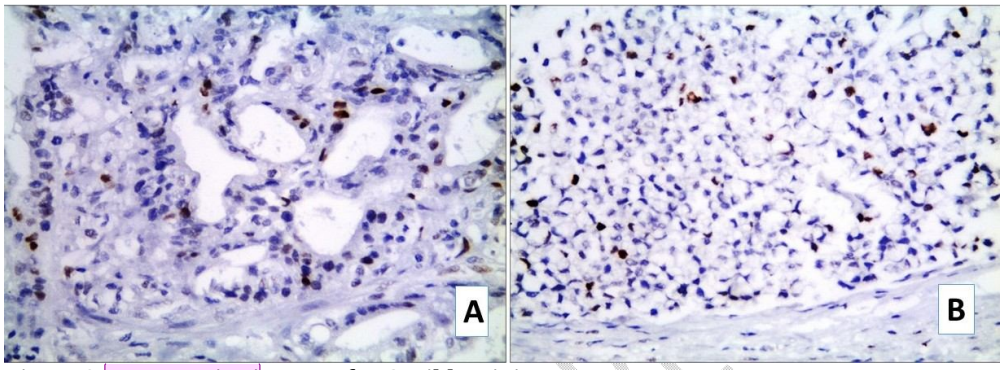

Figure 8:Representive images of p53 wild staining patterns:

A: A case of moderately differentiated tubular adenocarcinoma, P53 wild type (streptavidin biotin x400).

B: A case of poorly cohesive carcinoma (signet ring type), P53 wild type (streptavidin biotin x400).

Relation between P53 type and clinicopathological characteristics of the studied cases

Relation between P53 type and clinicopathological characteristics of the studied GC cases is summarized in**table 6**. The relation between P53 type and tumor differentiation of the studied cases was statistically significant with mostundifferentiated cases (28/46;60.9%) showing p53 aberrant type (p value=0.029).The relation between P53 type and lymph node status (N) was statistically significant with the majority of cases in N2 (14/20;70%) and N3 (8/10;80%) categories (more frequent lymph node metastasis) of p53 aberrant compared to P53 wild type (p value=0.006). A statistically significant relation between P53 type and TNM staging of the studied cases was found (p value<0.001), with most of stage III (18/22;81.8%) and stage IV (8/10;80%) (Advanced stage) cases classified as p53 aberrant type.

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Table 6: Relation between P53 type and clinicopathological characteristics of the studied gastric carcinoma cases

Clinicopathological variables	N.	P53 type		χ^2	P value
		P53 aberrant type	P53 wild type		
		N.(%)	N.(%)		
Age					
<60 years	29	17 (58.6)	12 (41.4)	1.025	0.311
≥60 years	41	19 (46.3)	22 (53.7)		
Gender					
Male	38	23 (60.5)	15 (39.5)	2.754	0.097
Female	32	13 (40.6)	19 (59.4)		
Tumor location					
Cardia and fundus	16	12 (75.0)	4 (25.0)	5.802	0.055
Body	10	6 (60.0)	4 (40.0)		
Antrum and pylorus	44	18 (40.9)	26 (59.1)		
Tumor size					
≤ 5 cm	20	10 (50.0)	10 (50.0)	0.023	0.880
> 5 cm	50	26 (52.0)	24 (48.0)		
Histopathological type					
Tubular	26	12 (46.2)	14 (53.8)	4.654	0.372
Papillary	4	2 (50.0)	2 (50.0)		
Poorly cohesive	26	12 (46.2)	14 (53.8)		
Mucinous	10	6 (60.0)	4 (40.0)		
Mixed	4	4 (100.0)	0 (0.0)		

Tumor differentiation					
Differentiated	24	8 (33.3)	16 (66.7)	4.78	**0.029***
Undifferentiated	46	28 (60.9)	18 (39.1)		
Depth of invasion					
T1	4	2 (50.0)	2 (50.0)	**11.257**	**0.008***
T2	20	6 (30.0)	14 (70.0)		
T3	38	20 (52.6)	18 (47.4)		
T4	8	8 (100.0)	0 (0.0)		
Lymph node status					
N0	18	4 (22.2)	14 (77.8)	12.490	**0.006***
N1	22	10 (45.5)	12 (54.5)		
N2	20	14 (70.0)	6 (30.0)		
N3	10	8 (80.0)	2 (20.0)		
TNM stage					
Stage I	6	2 (33.3)	4 (66.7)	21.136	**<0.001***
Stage II	32	8 (25.0)	24 (75.0)		
Stage III	22	18 (81.8)	4 (18.2)		
Stage IV	10	8 (80.0)	2 (20.0)		
Vascular invasion					
Absent	36	16 (44.4)	20 (55.6)	1.447	0.229
Present	34	20 (58.8)	14 (41.2)		
Perineural invasion					
Absent	51	23 (45.1)	28 (54.9)	3.014	0.083
Present	19	13 (68.4)	6 (31.6)		
MS status/ MMR status					
MSI/MMR-deficient	15	5 (33.3)	10 (66.7)	2.502	0.114
MSS/MMR-proficient	55	31 (56.4)	24 (43.6)		

***P value less than 0.5 was considered statistically significant**

Relation between MS status/MMR status and P53 type of the studied cases

Most MSI/MMR-deficient cases (10/15; 66.7%) were P53 wild type. However, the relation between MS status/MMR status and P53 type of the studied cases didn't reach a statistically significant value.

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Discussion

GC is the world's fifth most frequent cancer, behind lung, breast, colorectal, and prostate cancer. In terms of total mortality, it is the third greatest cause of cancer deaths globally, after only lung and colorectal cancer[22]. In Egypt, GC is the 12th most prevalent cancer in both sexes, accounting for 1.6% of all cancers, and it is the 12th greatest cause of cancer death, accounting for 2.2% of all cancer deaths[23].

The molecular classification of GC was a big step forward since it represents tumor biology and may be linked to specific clinicopathological facts[24]**.**It is deemed critical for GC diagnosis [25]and selecting targeted treatments[11].The most thorough molecular categorization of GC was published in 2014 by TCGA Research Network. They suggested a molecular categorization that subcategorized GC into four subtypes: tumors with MSI, CIN, tumors positive for the EBV, and tumors with GS cancers [3].

Four molecular subtypes of GC were identified by ACRG in 2015: MSI, MSS/EMT, MSS/TP53+, MSS/TP53- which showed the greatest rate of TP53 mutation[4]. The TCGA and ACRG classification systems employed expensive and advanced high throughput technology. However, this sophisticated methodological approach may not be adopted into normal surgical specimen processing in the near future [26].

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It has recently been proven that molecular subtyping of GC is possible using immunohistochemistry (IHC) and in situ hybridization (ISH) [27]**.**Setia et al.,[28]offered a simplified and less expensive algorithm for GC molecular subtyping. They offered procedures often used in diagnostic practice, such as ISH and IHC, and found five GC groups that match TCGA and ACRG molecular subgroups: EBV+, mismatch repair-deficient, aberrant E-cadherin expression, normal p53 expression, and aberrant p53 expression [28]. The WHO (2019)

recommended that in conventional pathology, IHC of mismatch repair proteins (MLH1, etc.), p53, and E-cadherin coupled with ISH for EBV-encoded small RNA would provide molecular GC subtyping [6].

MMR is a highly conserved DNA repair mechanism that recognizes and repairs single-base mismatches that elude polymerase proof-reading activity [29]. Because of its active involvement in preserving DNA integrity, this mechanism is critical for cell homeostasis [30]. Tumors with MMR dysfunctions are more likely to be hypermutators [31].

Although PCR-based molecular testing has been considered a standard diagnostic method fordetectionof microsatellite instability, IHC is simpler, quick, and widely available than molecular testing. IHC showed a high concordance rate compared with PCR-based assays in detection of MSI. IHC demonstrated sensitivity of 91.1% and specificity of 98.5% compared to PCR-based analysis in the identification of MSI phenotype ingastric cancer [32]. Additionally, Kim et al.[33], stated that IHC achieved a similar level of specificity and sensitivity as PCR-based molecular testing of MSI.

The rationale for using IHC of MMR proteins for assessment of MSI was mentioned in previous reports as follows: IHC of MMR proteins has high sensitivity andspecificity in addition to positive and negative predictive values for the mismatch repair system deficiency [28,34]. Furthermore, the main mechanism of microsatellite instability in gastric cancer is promoter hypermethylation of MLH1, less commonly, mutations in MLH1 and MSH2 [35]. Moreover, MLH1 IHC detection has showed highly concordant results compared to PCR assessment[36].As a result, IHC may be used to test for MMR deficiency in GCs in a straightforward and reliable manner.

Given the reliability, cost-effectiveness of IHC, availability of MMR antibodies in pathology laboratories, and that most cases show straightforward interpretation, this method (IHC) is widely considered as a first-line diagnostic test[37–39].

In agreement with Bae et al.[32], Kim et al.[33], Inada et al., (2015) &Giampieri*et al*.[9]The present studyidentified MMR-defective tumors based on the lack of MLH1 or MSH2 expression, regardlessof the expression of MSH6 or PMS2in the tumor. Although MSH6 and PMS2 expression are well known to be important factors in the definition of MMR activity in other tumors (such as colon cancer), it seems in GCthat these two genes are less relevant, with MLH1-defective status being the alteration most frequently found in MMR-defective tumors.

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In the current study, 21.4% of the studied gastric adenocarcinoma cases were classified as tumors with microsatellite instability (MSI)/mismatch repair deficient (MMR-deficient) cases while 78.6% of cases were classified as microsatellite stable (MSS)/ MMR-proficient cases.This frequency was consistent with previous reports [40–42]but higher than [43-45] and lower than other studies[46,47].

The discrepancies between the different studies may be explained by the variability in population size and population characteristics, variability of antibodiesand differences of interpretation of expression profiling (variation of the scoring system or the cutoff levels used).

Other cause of discrepancy is using whole surgical section versus tissue microarray.In agreement with Bosch et al.[5], and Di Pinto et al.[43], the current investigation used ICH on the whole section of surgical GC specimens, while most previous studies used tissue microarrays (TMAs), which have been proven not to be accurate representations of intratumor heterogeneous protein expression [27,28,48,49]. As a result, assessing the whole tumor region is critical for a more precise assessment of the MMR status.

The present study aimed to correlate the MS status/MMR status of the GC-studied cases with clinicopathologic variables. As regard to the histopathological type of gastric carcinoma, the majority of previous studies were not concerned with WHO histopathological subtype, but rather withLauren's classification and Lauren's intestinal-type histology predominated in microsatelliteunstablegastric carcinomas [1,5,14,27,28,32,41,49,50].

In the current study, we studied the relationship between WHO histopathological subtypes of gastric carcinomas and microsatellite status to examine histological diversity associated with MSI. The relation between MS status/MMR status and WHO histopathologic types of the studied gastric adenocarcinoma cases was statistically significant with all papillary adenocarcinoma cases showing MMR deficiency (MSI). Similarly, Sugimoto et al.[46], reported that the relation between MSS and histological type of tumor (Papillary versus Non-papillary) was statistically significant with most papillary adenocarcinoma cases showing MSI-high phenotype and Arai et al[51]., stated that the proportion of MSI is significantly correlated with certain histological subtype (such as papillary adenocarcinoma and solid-type poorly differentiated adenocarcinoma). This finding was inconsistent with previous reports [47,52]. Additionally, Setia et al. reported that MSI-gastric cancer subgroup included multiple WHO

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patterns but carcinoma with lymphoid stroma was significantly associated with microsatellite instability compared to other molecular subtypes[53].

It has been hypothesized that papillary adenocarcinoma is a separate morphological form with a molecular pathway of MSI since it has been discovered that microsatellite-unstable papillary carcinoma exhibits preferential hypermethylation of the hMLH1 promoter, with an absence of hMLH1 expression, and frequent mutations in genes such as BAX, MSH3, and MSH6. In early GC, the papillary type was the one that revealed centromere numerical abnormality[51].

In the present study, a statistically significant relation between MS status/MMR status and tumor differentiation was found in the studied cases with the majority of cases with MSI were of differentiated type which was consistent with Bae *et al*.[32], Park *et al*.[14],and Verma*et al*[47] who reported that MMR deficiency (MSI) was significantly associated with more differentiated histology (compared to MMR-P/MSS cases) and Ahn *et al*. [49] who reported significant association of high-MSI tumors with better-differentiated histology (compared to other molecular subtypes).

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On the contrary, previous reports showed no significant association between MMR and histological differentiation/tumor grade[45,50,54] and Bösch*et al*. reported a significant association of different molecular subtypes with tumor grade with both MMR/MSI and aberrant p53 subtypes were more associated with increased G3 probability (poorly differentiated)[5].

Arai et al. [51], showed no significant difference in the frequency of MSI between the differentiated and the undifferentiated type of GC. However, they reported that poorly differentiated carcinomas were significantly less common than differentiated carcinomas in microsatellite-unstable cancer at the early stage, whereas no significant difference found at the advanced stage. They proposed that gastric carcinoma with MSI develops principally as a differentiated-type tumor then progresses to a more poorly differentiated tumor (at the advanced stage).

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The present study found an insignificant relation between MS status/MMR status and depth of tumor invasion (T) which was consistent with previous reports [47,50,54,55]. On the contrary, Dislich et al.[1]& Ito et al. [45] reported that d-MMR/MSI-H cases were significantly associated with shallower tumor depth (lower pT-category) compared to MMR-P/MSS cases.

MMR deficient cases in the present study were significantly associated withlower rate of lymph node metastasis compared to MMR proficient cases. This finding was in line with previous reports [1,45,50,55]. Besides, Setia et al. [28], reported that MSI subtype was significantly associated withdecreased rate of lymph node metastasis (lower pN stage) compared to other molecular subtypes. This finding also reflects lesser biologic aggressiveness and a trend towards longer survival in this group. Additionally, Arai *et al.*[51], reported a statistically significant relation between MSI and lower rate of lymph node metastasis at advanced stage but no significant relation was detected in early stage compared to MSS cases. On the contrary, MMR deficiency/MSI was not significantly associated with lymph node metastasis in other studies[18, 47,52,54]

In the present study the relation between MS status/MMR status and TNM staging of the studied gastric carcinoma cases was statistically significant with the majority of cases with d-MMR/MSI were of stage I & II (early stage) which was consistent with previous studies [32,41,45,50,52,56]. Conversely, This finding was inconsistent with other studies [18,43,55].

The present study revealed an insignificant relation between MS status/MMR status and vascular invasion in the studied gastric adenocarcinoma cases. This was in line with previous studies [18,47,54]. On the contrary, Park *et al.*[14] reported that MSI-H was significantly related to presence of lymphovascular invasion (LVI) compared to MSS cases and Ito et al.[45] reported that d-MMR cases were significantly associated withmore frequent presence of venous invasion (but not significantly associated with lymphatic invasion) while Tsai et al. stated that d-MMR cases were significantly associated with less frequent vascular invasion (but not significantly associated with lymphatic invasion) compared to MMR-P cases[50].

The relation between MS status/MMR status and perineural invasion in the studied cases was statistically insignificant in the present study. This finding was consistent with[55,57]and inconsistent with previous studies[50,54]

One weakness of our research is the absence of long-term follow-up, which results in a lack of overall survival statistics. However, the comprehensive pathological variables that are accessible show a prognostic relationship between patients with MMR-deficient tumors and a better disease course. This is indicated by a lower frequency of lymph node metastases, a lower tumor stage, and better differentiation, all of which represent a lesser biologic aggressiveness. These observations are consistent with the previously published studieswho reported that

dMMR/MSI carcinomas have the best survival and the best prognosis between all GC molecular subtypes[44,56,58,59].Additionally, Bae *et al*.[32]and Zhang *et al*[60] also reported that patients with dMMR phenotype had improved survival and better prognosis compared to those with pMMR status.

The considerable T cell infiltration in these tumors may explain the better survival of individuals with MSI gastric cancer. Indeed, frame-shift mutations that produce aberrant peptides that may be delivered to cytotoxic T cells are common in MSI malignancies [59,61].

Ramos *et al.* [56]reported that MSI GC was the subtype withhighestsurvival rates. It's still unclear why tumors associated with MSI commonly have better prognosis. The lymphocytic peritumoral infiltration in these types of tumors, in response to peptides produced by MSI tumors, is thought to contribute considerably to the anti-tumor response by triggering tumor cell death through cytokine activation[62]. The lower LN involvement, which is a hallmark of this subtype in addition to other characteristics of these tumors,can also be related to their favorable prognosis. Even in situations when the pN status is positive, metastases often only impact a limited number of LNs[63]. Moreover, Martinez-Ciarpaglini et al.[18] stated that in multivariate analysis, MSI was substantially linked to a lower risk of death, regardless of other variables such as clinical stage or nodal metastases. Due to the prognostic significance of MSI testing in GC patients, we advise that it be used in clinical practice. Furthermore, MSI may provide usefulinformation to avoid neoadjuvant chemotherapy in cases with limited locoregional disease or to consider immunotherapy in relapsing or advanced-stage cases [57].

On the contrary, Arai *et al.* [51] demonstrated that there was no significant difference in prognosis between MSS and MSI gastric cancersandKim *et al.* [64]reported that tumors with MSI appear to be linked to worse prognosis.

The transcription factor p53 is a key tumor suppressor protein in humans, which is mutated in more than half of all human malignanciesalong their progression. Mutant p53 is regarded as a promising and unique drug target in the field of cancer therapy because of its frequent involvement in cancer progression[65,66]

Normal p53 [wild type] have short half-life that cannot be stained [67]. The majority of TP53 mutations are missense mutations, and IHC results demonstrate that p53 is overexpressed as a result of the buildup of non-functional proteins [42]**.** Proteins with a longer half-life are produced by TP53 mutations, and since they build up in the nucleus, they are overexpressed.On

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the other hand, a truncated protein can result from some missense or point mutations that isnot stable enough to cause any noticeable nuclear accumulation. Therefore, the absence or increased p53nuclear expression can be used to identifyits aberrant expression [56,68]

Additionally, Grosser et al.[69] performed mutational analysis of the TP53 gene by next-generation sequencing (NGS) and a concordant result with aberrant p53 expression (including overexpression and complete loss) by IHC analysis were demonstrated in (90%) cases. Hwang et al.[70] stated that IHC results showed that TP53 missense mutations were strongly correlated with strong p53 expression, other kinds of mutations were negatively correlated with high p53 expression, and wild-type TP53 was correlated with weak p53 expression (p-value 0.001). Each category's sensitivity and specificity were respectively 90.9%, 79.0%, and 80.9% and 95.4%, 88.1%, and 92.3%. Therefore, p53 protein IHC can be used as a simple surrogate marker of TP53 mutations[69].

In a study that combined an IHC labeling pattern associated with TP53 mutations (0% and 60-100% positive cells) and a nucleotide sequence analysis of p53 in ovarian cancer, the researchers found that combining the two patterns correctly identified a mutation in 94% of instances (P 0.001). They came to the conclusion that IHC is a valid approach for determining if ovarian cancer had the TP53 mutation[71]. A three-tiered scoring system, comprising overexpression, total absence, and a normal or wild-type pattern in ovarian cancer, was suggested by Kobel et al. [72]for the interpretation of P53 IHC. The scoring method showed a strong correlation with the TP53 mutation status, showing overexpression in the presence of nonsynonymous mutations, total absence in the presence of stop gain, frameshift, and splicing mutations, and a typical pattern in the presence of the wild-type TP53 gene.

As regard gastric adenocarcinoma, it was recently demonstrated that immunohistochemical (IHC) staining (which is widely available technique in routine diagnostic practice) could be used as a surrogate for TP53 gene mutational analysis to approximate the results in a simple and cost-effective manner[27,49,70].As the CIN subtype of TCGA molecular classification (2014) showed the highest frequency of P53 mutations, therefore P53 protein aberrations by immunohistochemistry can be used as a surrogate for CIN subtype [5]

Ando *et al*.[67], stated that; only positive or negative p53 staining had no effect in determining the mutation status of TP53 gene in gastric cancer. They reported that; in positive p53 tumors (showing 10% or more nuclei stained with p53), two types of staining could be

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distinguished; aberrant type (staining of 70% or more nuclei) and scattered type (20–50% of nuclei stained) and they observed a significant relation between p53 staining pattern and TP53 gene mutations. It's interesting to note that all cancers with the dispersed tumor type had wild-type TP53 genes, and all tumors with missense mutant TP53 genes exhibited abnormal p53 staining[67].

Aberrant expression of p53 by immunohistochemistry has been assessed differently in GC: some studies rated tumor cells'p53 overexpression using different cutoffs [36][48], whereas others included the loss of p53 immunostaining as aberrant expression [28][49]. Daun*et al.* [73]combined p53 IHC, including loss and overexpression, and TP53 gene sequencing by NGS technique for *detection* of P53 aberrant cases.

Various studies have used various cutoffs to estimate p53 overexpression in tumor cells when assessing p53 aberrant expression in GC [36][48], while other research have considered the loss of p53 immunostaining as an aberrant expression [28][49]. For the purpose of identifying P53 aberrant expression, Daun*et al.* [73] coupled p53 IHC, including loss and overexpression, with TP53 gene sequencing using the NGS method.

Grosser *et al.*[69]& Kim *et al.* [74] defined tumors with aberrant p53 expression as tumors showing complete absence or diffuse and strong p53 nuclear positivity. In the current study, we interpreted strong diffuse nuclear staining in > 90% of tumor cells or complete absence of p53 expression in all tumor cells as aberrant expression of p53 otherwise; p53 expression was categorized as wild type as reported by Bosch *et al.*[5].

In the present study, 51.4% of the studied gastric carcinoma cases were classified as P53 aberrant type while 48.6% were classified as P53 wild type.

The relation between P53 type and tumor differentiation of the studied GC cases was statistically significant in the present study with the majority of cases classified as p53 aberrant type showing undifferentiated morphology which was consistent with Bösch*et al.*[5]who reported significant association of different molecular subtypes with tumor grade (both dMMR/MSI and aberrant p53 subtypes were more associated with an increased G3 probability/poorly differentiated).

On the contrary, Birkman*et al.*[27] reported no significant association between P53 expression and histological grade among intestinal-type tumors. Kim *et al.*[75] reported that P53 overexpression/null tumors (corresponding to P53 aberrant subtype) were significantly

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associated with more differentiated histology compared to p53 weak group and Ramos et al.[56] reported a statistically significant association between different molecular subtypes and histological grade differentiation with the p53 aberrant tumors were more frequently well or moderate differentiated tumors. Additionally, Ando *et al*.[67] reported that P53 mutation (detected by TP53 gene mutation analysis) showed a statistically significant association with tumor differentiation with TP53 gene mutation found more frequently in differentiated than in undifferentiated type.

The present study revealed that the relation between P53 type and depth of tumor invasion (T) was statistically significant with the majority of cases classified as p53 aberrant type showing T3&T4 categories (deeper tumor invasion). This was inconsistent with previous studies [5,27,56,73]

The relation between P53 type and lymph node status (N) was statistically significant in the present study with the majority of gastric carcinoma cases classified as p53 aberrant type showed N2&N3 categories (more frequent lymph node metastasis) which was consistent with Setia et al.[28] who reported that GC with aberrant p53 expression were significantly associated with higher lymph node stage compared to other molecular subtypes and Grosser et al.[69], who reported that aberrant p53 expression was associated with a positive lymph node status. On the other hand, the relation between P53 expression and lymph node extension was statistically insignificant in previous studies [5,56,67].

The present study revealed a statistically significant relation between P53 type and TNM staging of the studied gastric carcinoma cases was with the majority of cases classified as p53 aberrant type showing stage III & stage IV (advanced stage) which was consistent with Hwang et al. [70] who reported that; the TNM stage at thefirst diagnosis showed a significant correlation with p53 expression status with most cases showing strong P53 expression were of stage III. This finding was inconsistent with others[27,44,56,67,75]

A limitation of the present study is the absence of prolonged follow-up and absent overall survival data. However, the available pathological parameters show a prognostic association between patients with P53 aberrant tumors and poor prognostic parameters indicating a more aggressive disease course, with deeper depth of tumor invasion, more frequent lymph node metastases, higher tumor stage and more undifferentiated histology which reflects more biologic aggressiveness of P53 aberrant tumors compared to P53 wild type tumors.

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Similarly, Grosser *et al.[69]* reported that aberrant p53 expression was significantly associated with worse overall survival in the total resected tumor cohort. The ACRG [41]Bösch*et al*.[5] Pinto *et al*.[44]&Nshizirungu*et al*.[59] reported that P53 aberrant carcinomas have intermediate prognosis and survival compared to other molecular subtypes of GC. Ahn *et al*.[49]& Kim *et al*.[64] reported that MSI tumors had the best prognosis, followed by the EBV tumors; cases with normal p53 expression, aberrant p53 expression, and EMT/GS tumor showing the worst prognosis. Gurzu*et al*.[76] reported that TP53 wild-type cases had a longer survival, compared with P53 mutant GCs but didn't reach a statistically significant value. On the contrary, no significant survival difference was noted in GC with aberrant p53 expression compared to the remaining adenocarcinomas in other studies [27,28]

The present study revealed that most MSI/MMR-deficient cases (66.7%) were p53 wild type but the relation between MS status/MMR status and P53 type of the studied cases didn't reach a statistically significant value. Similarly, Gonzalez *et a*l.[48] stated that TP53 mutation can occur in cancers associated with MSI, though there is no statistically significant link of MSI and P53 aberrant type cases.

On the other hand, Birkman*et al*.[27] reported a significant association between P53 expression and microsatellite status with TP53 aberrant tumors were more frequently MSS than MSI. Hwang *et al*.[70] reported that p53 expression showed a significant association with MSI status with all p53 aberrant cases (strong P53 expression and negative P53 expression)of the MSS/MSI-L type. Additionally, Grosser *et a*l.[69] also reported a strong association of aberrant p53 with MSI-L.

Setia et al.[28]stated that MSI GC have been identified as a distinct group based on clinicopathologic and molecular characteristics. Yet, this subset may have aberrant p53 expression along with TP53 anomalies (most frequently because of loss of heterozygosity).

Conclusions:MSI phenotype GC and P53 aberrant GC are distinct molecular subtypes of gastricadenocarcinoma with distinct clinicopathological features and differentprognostic outcomes.MSI/MMR-deficient gastric tumors are associated with good prognosticparameters with a lower frequency of lymph node metastases, lowertumor stage and better differentiation which reflects lesser biologicaggressiveness compared to MSS/MMR-proficient tumors.P53 aberrant gastric tumors are associated with poor prognostic parameters withdeeper depth of

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tumor invasion, more frequent lymph node metastases,higher tumor stage and more undifferentiated histology which reflectsmore biological aggressiveness of P53 aberrant tumors compared to P53wild-type tumors.

Recommendations: Further studies should be carried out to investigate the patients' survivalin MSI GC and P53 aberrant GC.Large scale studies including large number of GC patients could be usedto validate the findings of the present study.Different molecular subtypes of gastric adenocarcinoma with distinctmolecular characteristics can represent potential targets for futuretherapeutic agents and further understanding of gastric cancerbiology.Further molecular studies should be carried out in the future seeking moremolecular properties that could be applied using immunohistochemicalstaining for better identification of different molecular subtypes of GC.

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