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Maspin subcellular expression of wild-type and mutant *TP53* gastric cancers

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ABSTRACT

BACKGROUND

Although the role of p53 in evolution and prognosis of gastric cancer (GC) has been examined extensively, the exact mechanism of action is incompletely understood. In the last few years it was believed that p53 target genes were involved in the p53 pathway. One of these genes is the tumor suppressor gene Maspin, which codes for the protein of the same name. Maspin activity depends on its subcellular localization. To the best of our knowledge, the possible role of *TP53* gene in Maspin subcellular localization in GC cells, has not yet been studied in a large number of human samples.

AIM

The aim of this paper was to evaluate the possible role of wild-type and mutated p53 in Maspin subcellular localization.

METHODS

The present study included 266 consecutive patients with GC in which *TP53* gene status, specifically mutations in exons 5 to 11, was analyzed using Sanger sequencing and was correlated with immunohistochemical (IHC) expression of p53 and Maspin.

RESULTS

None of the 266 cases showed mutations in exon 9. The rate of *TP53* mutations was 33.83%. The mutation rate was slightly higher in distally-located GCs with a lower degree (≤5 buds/HPF) of dyscohesivity (p=0.005). The wild-type cases proved to have a longer survival compared with mutant GCs, especially in patients without lymph node metastases despite a high depth of tumor infiltration (p=0.01). The Dukes-MAC-like staging system provided the most significant independent prognostic value (p=0.0006). The statistical correlations proved that *TP53* gene mutations in exon 7 might induce knockdown Maspin, but wild-type p53 can partially restore nuclear

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Maspin expression and decrease the metastatic potential of gastric adenocarcinoma cells.

CONCLUSIONS

Downregulated Maspin might be induced by mutations in exon 7 of *TP53* gene, but wild-type p53 can partially restore nuclear Maspin expression. This should be proved in experimental studies.

Keywords: p53, TP53 gene, Maspin, gastric cancer, carcinoma

Core tip: In this paper we tried to emphasize the possible prognostic role of *TP53* status in gastric cancer and its relationship with Maspin protease. We have proved that *TP53* gene mutations in exon 7 might induce knockdown Maspin, but wild-type p53 can partially restore nuclear Maspin expression and decrease the metastatic potential of gastric adenocarcinoma cells.

INTRODUCTION

In many cancers, including gastric carcinomas (GC), *TP53* is known to be frequently mutated[1,2]. Despite this well-known fact, the prognostic role of *TP53* is still controversial. Moreover, its interaction with other biomarkers is constantly checked, but the results are controversial.

It is known that *TP53* plays roles in genomic stability[1], but the mechanism of interaction with other p53 target genes such Maspin is unclear[2,3]. Maspin (Serpine B5) is a serine protease that is known to be involved in the tumor cell proliferation and exerts anti-angiogenic and anti-apoptotic properties against tumor cells[2-4]. Its regulatory functions depend on the subcellular localization (nucleus versus cytoplasm)[3,5].

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In this paper, we examined the p53 and Maspin immunohistochemical (IHC) expression in GC samples, correlated with the mutation rate of *TP53* gene. The independent prognostic role of these markers was also checked.

MATERIALS AND METHODS

Patients and tissues samples

The present study was performed taking into account 266 GC cases, which were retrospectively collected from the Clinical County Emergency Hospital of Targu-Mures, Romania. The Ethical Committee of the Clinical County Emergency Hospital, Targu-Mures, Romania, approved retrospective evaluation of the cases.

In our patient sample, surgical intervention was performed between 2006 and 2016. It consisted of partial or total curative gastrectomy with lymph node dissection. No cases with stump carcinoma, associated peptic ulcer or patients receiving preoperative chemo or radiotherapy were included.

Clinicopathological assessment

In the 266 patients, the age and genders were correlated with tumor characteristics such as localization, stage, macroscopic type, microscopic type and histologic grade of differentiation, and with presence/absence of associated intestinal metaplasia. The tumors were macroscopically categorized based on the Borrmann's classification and criteria proposed by the Japan Gastroenterological Endoscopy Society, as type I - polypoid; type II - ulcerated; type III - ulcero-infiltrative; and type IV - diffusely infiltrative.

Histological diagnosis

All of the examined samples were surgical specimens. We did not include biopsy samples. The histopathological particularities were checked on carcinoma samples

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and classified based on the combined Lauren and World Health Organization tumor classifications[6]. The following two main histologic subtypes were included[6]. The first type were differentiated intestinal GCs: well-differentiated (G1), moderately differentiated (G2), poorly differentiated/papillary carcinoma (G3). The second type were diffuse GCs (non-mucinous poorly cohesive, signet ring cell carcinoma and microglandular GCs, defined as diffuse proliferation of small tubular structures).

Based on the rules used for colorectal carcinomas[5,7] which were adapted for GC[8], we also counted the grade of dyscohesivity (tumor budding degree). In the invasion front, we took into account at least five high power fields (HPF) and classified the cases into five groups: 0 – nodular growth intestinal type GCs (adenocarcinoma without buds); 1 – adenocarcinoma with 1-4 dyscohesive cells in the invasion front (low grade); 2 - adenocarcinoma with 5-9 dyscohesive cells in the invasion front (high grade); 3 - adenocarcinoma with more than 10 dyscohesive cells in the invasion front (high grade); and 4 – diffuse growth GCs.

Tumor staging

Although the cases were diagnosed from 2003-2016, they were re-staged, based on the two currently used systems. The first system was the 8th edition of the WHO/AJCC staging system[7]. The cases were classified based on the invasion of the mucosa (pT1a), submucosa (pT1b), muscularis (pT2), serosa (pT3) and crossing serosa (pT4). At the same time, the pN staging took into account the absence of lymph node metastases (pN0) and the number of involved lymph nodes (pN1-3)[7].

The second system used for the tumor staging was proposed by our team in 2017 and was called the Dukes-MAC-like staging system[9]. It consists of classification of GC

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based on the depth of invasion (T) and lymph node status (N), in eight groups, as follows: 1-T1N0; 2-T1N1-3; 3-T2N0; 4-T2N1-3; 5-T3N0; 6-T3N1-3; 7-T4N0; 8-T4N1-3[9].

Immunohistochemical stains

The immunohistochemical (IHC) stains were performed on the paraffin-embedded tissues. After deparaffinization, the tissues were processed with the Novolink Polymer Detection System (Novocastra, Newcastle upon Tyne, UK) according to the instructions of the manufacturer. We used the antibodies Maspin (clone EAW24, Novocastra, Newcastle upon Tyne, UK) and p53 (clone DO-7, LabVision, Fremont, CA, USA). The developing was done with DAB (diaminobenzidine) solution (Novocastra). For the negative controls, incubation was done with the omission of specific antibodies.

Interpretation of the immunohistochemical reactions

The IHC assessment was performed by two pathologists using the criteria previously described in the literature. For p53, three groups were considered based on the intensity and extent of stained tumor cell nuclei: negative (<5%), low (5-50%) and high p53 expression (>50%).

For Maspin, we used a system of quantification which was previously published by our team and based on the subcellular localization of this protease. Cases were classified as negative, with cytoplasm positivity, nuclear predominance and mixed expression (dual positivity in cytoplasm and nuclei)[3,4,5,10].

Molecular analysis

The DNA extracted from the paraffin blocks was used for genetic examination which was done at the Department of Tumor Pathology of Hamamatsu University School of Medicine, Japan. DNA isolation was done using the Qiagen kit following the

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manufacturer's protocol. *TP53* gene sequencing was carried out and gene mutations were checked in exons 5 to 11.

Follow-up and statistical analysis

Statistical analysis was performed using GraphPad Prims8 software. For descriptive statistics, the median value ± SD was used. Chi-square, ANOVA, Fisher's exact test and Spearman's test were used to check the correlations.

The median follow-up period was 36 months (range: 2–61 months). Patients who died in the first month after surgery were not included in the database. Univariate survival proportion was evaluated using Kaplan-Meier survival curves.

A p value of <0.05 (with a 95% confidence interval) was considered statistically significant.

RESULTS

Clinicopathological factors

In the present database, which included 266 patients with GC, we noted a predominance of males vs. females (M:F=1.9:1). The GC was diagnosed at the median age of 62.52±14.34 (range 22 to 98 years), in both males and females (p=0.84).

Up to 55% of the cases were ulcero-infiltrative adenocarcinomas of the proximal and middle third stomach, which were not developed on the background of intestinal metaplasia. Although in both young and elderly patients a similar distribution between proximal and distal stomach was observed, the Spearman's correlation showed that the predominance of involvement of the distal stomach was slightly increased in patients over 60 (p=0.04). In elderly patients, 109/266 cases (40.98%) were developed on the background of intestinal metaplasia, whereas only 47/266 (17.67%) of GCs of patients ≤60 years presented associated metaplasia (p=0.0006). In line with

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these results, metaplasia-related intestinal type carcinomas were more frequent in the elderly, compared with younger patients (p=0.0001).

Examination of the budding degree showed that over half the cases (62.03%) showed high-grade dyscohesivity or infiltrative growth. Nodular growth was found in only 12.78% of the cases (Table 1).

Only 37 out of the 266 cases (13.91%) were identified in early stages (pT1+2). Over 80% of the cases showed metastases in at least one lymph node, independently by the depth of tumor infiltration (Table 1).

Correlation of clinicopathological factors with p53 protein expression and TP53 gene status

From the 266 cases examined, 40 proved to loss the p53 expression. In the other 226 p53-positive cases, 157 showed nuclear expression in over 50% of the tumor cells (Table 2), without correlation with any of the examined clinicopathological factors (Table 3).

The *TP53* gene shows mutations in 90/266 (33.83%) cases, the other 176 (66.17%) being wild-type (wt) cases (Table 2). Independent of the mutated exon and other parameters, the statistical examination showed a slight predominance of the p53 wt cases for tumors of the distal stomach which also presented the highest degree (>5 buds/HPF) of dyscohesivity (Table 3).

Correlation of clinicopathological factors with Maspin protein expression

Subcellular expression of Maspin positivity was checked in both tumor core and invasion front. In the tumor core, it was observed that most of the cases expressed mixed expression (nuclear+cytoplasm) for Maspin, followed by cytoplasm and negative cases. In the invasion front, the Maspin subcellular expression was significantly modified compared with the core (p<0.0001) (Table 1). It was noticed that

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the nuclear expression was completely maintained (in the cases with nuclear Maspin in the core) and all of the cases with cytoplasm stain, in the core, gained nuclear expression in front. Some of the mixed cases lost the cytoplasm stain and other negative cases, in the core, showed nuclear positivity in the invasion zone.

The statistical correlations showed that in the tumor core, there was a predominance of cases with nuclear positivity in younger patients with poorly cohesive carcinomas, whereas Maspin cytoplasmic expression was most frequently seen in patients over 60 with intestinal-type adenocarcinomas with nodular growth or low grade dyscohesivity (≤5 buds/HPF). All of the "linitis plastica" cases showed pure nuclear Maspin in the invasion front (Table 4).

Correlation of immunohistochemical markers Maspin and p53 with TP53 gene status

The most interesting correlation was in relation to the Maspin subcellular expression in the invasion front. On the one hand, all of the 21 Maspin negative cases in both core and front (Table 1) showed mutations in exon 7 of the *TP53* gene (C>T and G>A). All of them were high-grade budding (2, 3) adenocarcinomas without associated intestinal metaplasia, which expressed p53 in over 50% of tumor cells and were diagnosed in stages T3,4N1-3. On the other hand, all of the 44 cases with nuclear Maspin in the invasion area were wt *TP53*. At the same time, 18/39 Maspin negative cases, where the tumor core gained nuclear positivity in the invasion area, did not present *TP53* gene mutations (Table 5, Figure 1). All of the cases (n=44) with pure nuclear Maspin in the invasion area (Figure 1) were wt *TP53*.

No statistical correlation was found between p53 immunostain (nuclear or cytoplasm) and *TP53* gene status (Table 5). From the 40 cases with p53 negativity, 11 (27.50%) showed mutations in exon 3 to exon 8; in two cases double mutations were seen in

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exon 3+4 and 5+6, respectively. At the same time, 21 of the 69 cases with low p53 positivity (30.43%) presented mutations in exons 2, 5, 6, 7, 8, 10, and 11; in one case both exon 5 and 6 were involved. Finally, 15 out of 157 cases with p53 positivity in over 50% of tumor cells (36.31%) in exons 3, 4, 5, 6, 7, 8, 10 and 11. Double or triple mutations were seen in five of the cases involving exons 3+4, 4+5, 4+8, 5+6, and 6+7+8 (Table 3). No mutations in exon 9 were identified.

Overall survival

In univariate survival analysis, any of the following clinicopathological parameters proved to have independent prognostic value: tumor localization (p=0.35), macroscopic (p=0.20) or microscopic aspect (p=0.54), associated metaplasia (p=0.54), lymph node status (p=0.39), budding degree (p=0.51). A slightly longer survival was proved for males younger than 60 years (Figures 2 and 3).

The most significant independent prognostic parameters proved to be tumor depth of infiltration – pT stage (p=0.001) and Dukes-MAC-like stage (p=0.0006) (Figure 4). No prognostic value was proved for IHC expression of Maspin in tumor core (p=0.16) or invasion front (p=0.55), even for p53 expression (p=0.81) (Figure 5).

Since the *TP53* gene status had an independent prognostic value, but was not statistically significant (p=0.09), we adjusted it for pTNM and Dukes-MAC-like stage (Figures 5 and 6). Regarding the depth of infiltration, in locally advanced stages (pT3-4), wt cases showed a significantly longer survival than those with *TP53* gene mutations (p=0.01); the difference was not significant in the group of patients diagnosed in early stages, specifically pT1-2 (p=0.68). Within the group of patients without lymph node metastases (pN0 and Dukes-MAC-like stage 1+3+5+7), the longer survival was also proved for wt cases, compared with those with *TP53* gene mutations

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(p=0.01). This fact was not available for patients with lymph node metastases. When these were examined independently, the pT stage parameter or combined TN (Dukes-MAC-like stage 1+3+5+7) had a p value of 0.51 (Figure 6).

Multivariate correlation indicated that negative prognostic value was associated with *TP53* gene mutations, especially in patients without lymph node metastases (Figure 6).

DISCUSSION

As the OS proved to vary in patients with GC within the same stage, the most recent papers proved that the current TNM staging system is not sufficient for prognosis estimation[1]. The present study confirms the utility of the newest Dukes-MAC-like staging system[9] for a proper estimation of long-term survival and to inform the independent prognostic role of IHC expression of Maspin and p53 in patients with GC. Despite the intention of including new biomarkers as having prognostic potential, the depth of invasion, combined with lymph node status (pTN stage) remains the strongest predictors of outcome of patients with GC[8,11].

This study confirmed the age-related particularities[11], respectively increasing number of distally-located GCs, especially adenocarcinomas developed on the background of intestinal metaplasia. However, the clinicopathological features, including grade of dyscohesivity (budding degree), did not prove to exert independent prognostic value.

In previously published papers, the presence of *TP53* gene mutation was not proved to be an independent prognostic factor for patients with advanced GCs[1,12], nor was it correlated with a shorter survival[13,14]. Our study confirmed the longer survival of wt cases compared with those with *TP53* gene mutation, especially in patients without lymph node metastases.

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The *TP53* mutation rate was proved to be dependent on various parameters, including geographic particularities, known to be induced by interactions between *Helicobacter pylori* infection with other environmental, molecular and genetic factors[13,15,16]. In our study, which included Romanian patients, the mutation rate was 33.83%. This is similar to American Caucasian patients (40%) and Hispanic patients (43%), whereas Asian, African American and Bangladeshi patients proved to have significantly elevated rates, 56%, 89%, and 73% respectively [16,37].

The literature shows a prevalence of *TP53* somatic mutations (over 95%) in exons 5 to 8, independently from the carcinoma localization[12,14]. As no correlation was seen between the mutation status and IHC expression of p53 protein, this study confirms that the p53 negativity cannot be used as an indicator of wild-type status[1]. However, this aspect is controversial. Some authors maintain that certain *TP53* somatic mutation types might associate p53 negativity and, as wild-type p53 protein has a very short half-life, it cannot be detected properly using IHC stains and false negativity can be obtained[12,17]. On the other hand, the clone DO7, also used in the present study, can only detect truncated mutations in exons 9 and 10[12,17].

Interaction of p53 with Maspin, previously proved for colorectal, prostate and bladder cancer[10,18,19], was partially confirmed in this study for GC.

In colorectal cancer, the experimental studies showed that, although Maspin nuclear positivity might be an indicator of aggressive behavior, it also indicates the possibility of responding to 5-Fluorouracil (5-FLU)-based chemotherapy. Most of the colorectal carcinoma cells displaying cytoplasmic and carcinomas with microsatellite instability and nuclear Maspin, proved to be p53 negative[4,20]. On the other hand, Maspin

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negative/p53 positive colorectal carcinomas are 5-FLU-resistant and have a risk for distant metastases[4,10,18,20]. The best prognosis was proved for p53 negative cases that displayed cytoplasmic positivity for Maspin, whereas Maspin nuclear staining associated with a p53 index of over 50% was an indicator for the worst prognosis[4].

In bladder and prostate cancer, p53 proved to upregulate Maspin expression and stimulate cisplatin-induced apoptosis[19]. Knockdown Maspin in p53 wild-type carcinoma cells stimulates tumor cell proliferation[19].

Although it was shown that two p53 binding sites are responsible for promoting the human Maspin gene: GGCATGTTGGAGGCCTTTG and GGACAAGCTGCCAAGAGGCTTGAGT[2,19,21], no relevant data about Maspin-p53 interaction were published in the field of GC. The present study suggests that knockdown Maspin might be induced by G:C→A:T transition in exon 7 of the *TP53* gene. As Maspin negative GCs show high risk for distant metastases[3], the role of exon 7 in GC behavior, especially in cases without lymph node metastases, should be more extensively explored.

Another unusual finding is the absence of *TP53* gene mutations in cases with nuclear Maspin positivity in the tumor front, known to present risk for local relapse and lymph node metastases[3]. Nuclear positivity was present in front and core in only 17/44 positive cases and over 46% of negative cases in core displayed Maspin nuclear expression in the invasion area only. In cell cultures, wt *TP53* proved to induce chromatin changes and even partial Maspin restoration, approaching basal levels of non-tumorigenic cells[2].

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Previous studies, including our own, showed that in GC, Maspin is downregulated in carcinoma cells compared with normal mucosa, and silencing of Maspin increases metastatic behavior[2,3]. The present study showed that Maspin silencing occurs more frequently in cases with mutations in exon 7 of the *TP53* gene, but wt p53 may induce changes in chromatin architecture and reactivate nuclear Maspin in the invasion front, decreasing the risk of distant metastases. The small number of cases do not allow interpretation of the relationship between p53 and mixed Maspin in which, it is likely that only partial Maspin restoration is obtained.

The in-depth exploration of the status of the *TP53* gene (one of the most frequently mutated tumor suppressor genes), proved to influence the GC behavior. In line with other studies, *TP53* mutations independently form the involved exon and were observed to increase the capacity of tumor invasion[1].

ARTICLE HIGHLIGHTS

Research background

Maspin is a serine protease that was extensively studied by our team using immunostains in GC and colorectal cancer samples. Our previous data, which are in line with the literature, showed that the role of Maspin is strongly dependent on its subcellular expression. In GC cells, Maspin downregulation increases the metastatic potential, cytoplasmic localization induces a better prognosis and nuclear stain is correlated with a higher local re

Research motivation

As data regarding the correlation between Maspin and p53 are scarce for GC, the aim of the paper was to check the particular features of this possible interaction. Of

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We compared the IHC stains for p53 and the p53 gene-related protein Maspin in GCs and correlated the results with *TP53* gene expression profile. The independent prognostic value of the examined parameters was also checked.

Research methods

In 266 consecutive GC samples, we performed IHC stains with the antibodies Maspin and p53 and performed Sanger sequencing (form paraffin-embedded tissues), to check *TP53* gene mutations in exons 5 to 11.

Research results

In the examined cohort, the *TP53* gene mutation rate was 33.83%, without correlation with the immunoexpression of p53 protein. The wild-type cases, especially those without lymph node metastases, showed a longer survival rate. The most significant independent prognostic parameter proved to be the Dukes-MAC-like tumor stage. The statistical correlations proved that Maspin nuclear restoration in the invasion front can be obtained in TP53 wild-type cases, whereas mutations in exon 7 of *TP53* gene induces Maspin negativity in both tumor core and invasion area.

Research conclusions

Despite several prognostic parameters proposed for GC, the survival rate is better predicted by the classic TN stage. Downregulated Maspin might be induced by mutations in exon 7 of *TP53* gene, but wild-type p53 can partially restore nuclear Maspin expression.

Research perspectives

Since this study is the first demonstration of the possible role of mutations in exon 7 of the *TP53* gene in downregulation Maspin, further investigations are necessary to elucidate the possible therapeutic role of anti-Maspin chemical derivates.

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