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ORIGINAL ARTICLE

Correlation of P53 Expression with Various Clinicopathological Parameters of Gastric Carcinoma and Its Relationship with Survival

Tushar Hiralal Sankalecha, Sudhir Jagdish Gupta, Nitin Rangrao Gaikwad, Suresh Narayanrao Ughade

Tushar Hiralal Sankalecha, Sudhir Jagdish Gupta, Nitin Rangrao Gaikwad, Suresh Narayanrao Ughade, Government medical college and super speciaity hospital, Nagpur, India
Tushar Hiralal Sankalecha, Senior resident, Department of gastroenterology, Government medical college and super speciality hospital, Nagpur, Maharashtra, India

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Correspondence to: Tushar Hiralal Sankalecha, Senior resident, Department of gastroenterology, Government medical college and super speciality hospital, Nagpur, Maharashtra, India
Email: dr.tushar30@gmail.com
Telephone: +91-0712(2750121-3) ext 260
Fax: +91-0712-2746682

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ABSTRACT

AIM: Gastric cancer is the most aggressive type of cancer. The immunohistochemical protein expression of mutant p53 has been proposed as a potential tool to evaluate the biological behavior of gastric cancer. Predictive value of p53 for survival is debatable; hence this study was formulated to know the survival of patients with p53 expression in gastric cancer.

METHODS: It is prospective study from September 2014 - July 2015, included 58 consecutive patients of gastric cancer. Biopsy specimens were treated immuno-histochemically and expression of p53 gene was analyzed by Immunoreactive Score (IRS). These findings were then compared with clinico-pathological parameters like age, gender, tumor location, tumor size, Laurens classification and TNM staging according to American joint committee for cancer guidelines, using CT scan of abdomen, and histopathological grading and types according to WHO classification.

RESULTS: Mp53 expression was observed in 90% of gastric cancer patients among which 37 (63.8%) patients showed high and 21 (36.2%) patients showed low p53 expression. Level of p53 expression was found significantly associated with age, tumor site, tumor size, histological grade, T stage, M stage and Clinical stage. Multivariate analysis shows that high p53 expression is an independent predictor of survival. On Kaplan-Meier survival analysis, patients with p53 high expression had significantly shorter overall survival than those patients with low p53 expression.

CONCLUSION: Expression of p53 correlates with the survival and is a simple, effective and reproducible modality to determine the prognosis and survival in various grades & stages of gastric cancer.

Key words: Gastric cancer; Gene expression; Immunohistochemistry

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INTRODUCTION

Gastric cancer is one of the most aggressive cancers worldwide. Gastric cancer is the fourth most frequent cancer and is second most leading cause of cancer related deaths worldwide. Adenocarcinoma of the stomach is the second and fourth most common cancer in males and females respectively[1,2]. The incidence of gastric cancer

varies in different parts of the world, with highest rates documented in Eastern Asia, Eastern Europe, and South America and lowest rates in North America and Africa[3-6]. In India, incidence of gastric carcinoma is higher in southern and north-eastern states[7,8]. About 95 % of gastric tumors are epithelial in origin and are designated as adenocarcinoma[9]. Prognosis of gastric cancer is poor as most of patients generally consult health care services in advanced stage of disease. Furthermore, surgery and chemotherapy have limited value in advanced disease. Prognosis and survival depends on early diagnosis and treatment. So there is need for specific histological and biological markers in order to identify the subgroups of patients with more aggressive course of illness in the same stage of disease. Many molecular alterations occur in gastric cancer and further investigation in to these alterations may provide clues to discover novel markers for improving diagnosis and guiding targeted therapy.

Many such potential markers were studied viz. Ki67, HIF, E cadherin, MMP-1, TGF-B, STAT3, TIMP1, HER2 in gastric cancer but amongst all, p53 was studied considerably[10-14]. The immunohistochemical protein expression of p53 has been proposed as a potential tool to evaluate the behavior biologically. Majority of studies suggest prognostic significance of p53 expression in gastric cancer, however some studies fail to show its role in gastric cancer[15-17]. This conflict in opinion led us to formulate the study with the aim of assessing the yield of p53 expression in gastric carcinoma and its relationship with survival.

MATERIAL AND METHOD

Patients

It is prospective study, carried out in the Department of Gastroenterology, Government medical college and Super Specialty Hospital, Nagpur, India. The Study Protocol was approved by institutional ethics committee. Study includes 58 consecutive patients of gastric cancer diagnosed by endoscopy (Figure 1A) and histopathology (Figure 1B) from September 2014 to July 2015. Biopsy tissue specimens were embedded in paraffin after fixation in formalin and were sent for immunohistochemistry. The following parameters were evaluated: age, gender, tumor location, tumor size, Laurens classification, TNM staging [according to AJCC Guidelines] [18,19], using CT scan of abdomen, and histopathological grading and types according to WHO classification[20, 21]. All patients received standard of care treatment according to stage of the disease. Patients were followed prospectively for 12 months and / or death from the date of diagnosis.

Immunohistochemistry

Immunohistochemistry was performed on tissues fixed in 10% neutral buffered formalin. The sections were cut serially to 5 µm for immunohistochemical staining. Peroxidase Detection System (Streptavidin-Biotin Detection System HRP-DAB; Product Code: RE7110K, Novo- castra kit) was used. Endogenous peroxidase activity was blocked by treating hydrated sections with 3% H_2O_2 in methanol for 30 min. The slides were heated in a microwave oven for 10 min in 0.01M sodium citrate buffer (pH6.0) for antigen retrieval and then bench cooled for 20 min and again the same cycle was repeated. To prevent non-specific reactions, sections were incubated with 10% serum for 10 min. Pre- diluted p53 antibody (clone DO-7; Product code: N1581, Dako, Denmark) was incubated at room temperature in a humidifying chamber for 60 min and then at 4°C overnight. Known tissues of carcinoma showing good p53 expression were used as a positive control. This was followed by incubation with

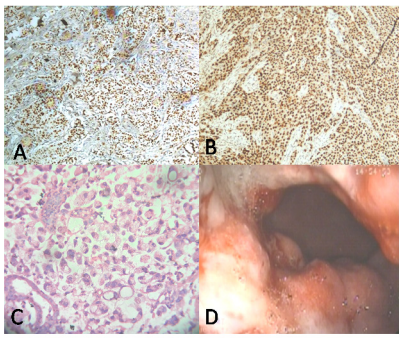


Figure 1 (A): Gastric malignancy with low expression of p53; (B): Gastric malignancy with high expression of p53; (C): Histopathology of gastric malignancy; (D): Endoscopy showing gastric malignancy.

secondary biotinylated antibody and streptavidin-peroxidase reagent at room temperature in a humidifying chamber for 30 min. Freshly prepared substrate/chromogen solution of 3, 3' Diaminobenzidine (mixing 5 ml of concentrated DAB in 50 ml of substrate buffer) was used to detect the antigen–antibody reaction. Finally, the sections were counterstained in Mayer's hematoxylin[22].

The IHC staining of mutant (MT) p53 was assessed according to the immunereactive score (IRS) [Table 1A, 1B], which is based on the percentage of positive cells and the staining intensity. The cells were considered positive for p53 antigen when there was an intra-nuclear DAB staining (brown color) [Figure 1C, 1D]. The percentage of positive cells were assessed with the help of labeling index (P53 Labeling index = Number of IHC Positive Cells X 100/ total number of cells observed). The two scores were multiplied to get IRS score, ranging from 0 to 12 and corresponded to ≤6 as low and >6 as high groups of p53 expression. The counting was done by two observers and the mean was taken as a final count.

Statistical analysis

The Statistical Package for the Social Sciences software version 20 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The χ^2 test and Fisher's exact test were performed to evaluate the correlation between the clinicopathological features of the patients and the p53 expression level. For the survival analysis, the Kaplan-Meier method with log-rank test was used. Prognostic factors were further evaluated in univariate and multivariate logistic regression analysis using the Cox's proportional hazards model to know relevant prognostic variables. The risk ratio (RR) with 95% confidence interval (95% CI) was used to assess the relationships between those factors and overall survival. A P value < 0.05 was considered as statistically significant.

RESULTS

Total 58 patients of gastric cancer were enrolled in our study. 38 (65.5%) were male and 20 (34.5%) were female with M: F ratio 1.9: 1. Age range in study population was 35-80 years with mean age of 59.63 (SD ± 6.2) years. Mp53 expression was observed in 90% of gastric cancer patients. Among the total of 58 patients 90% of patients show positive Mp53 expression and remaining 10% of patients show no expression. According to IRS scoring system 0 to 6 score is considered under low expression group. Therefore the 10% of patients showing no expression are considered under low expression group as per IRS system. Therefore low expression group numbers were up to 21 and high expression group numbers up to 37 patients.

Level of p53 expression was found significantly associated with age, tumor site, tumor size, histological grade, T stage, M stage and Clinical stage, where as it was not associated with gender, N stage, Lauren classification and histopathological type of tumor (Table 2A, 2B).

On Kaplan-Meier survival analysis, patients with high p53 expression group had significantly shorter survival than patients with low p53 expression group (log-rank $P < 0.00001$). (Figure 1) After 12 month of follow up, 56.76% (CI 0.39-0.70) of patients with p53 high expression group and 95.24% (CI 0.70-0.99) patients with p53 low expression group were alive. Multivariate analysis by Cox regression model further showed that high p53 expression was independent predictor of overall poorer survival (HR = 9.34; 95% CI 1.003-90.90, $P = 0.049$). However, gender, tumor location, tumor size, histological grade, histopathological type, lauren classification, T stage, N stage, M stage and clinical stage were not significant predictors of survival in gastric cancer patients (Table 3 and 4).

DISCUSSION

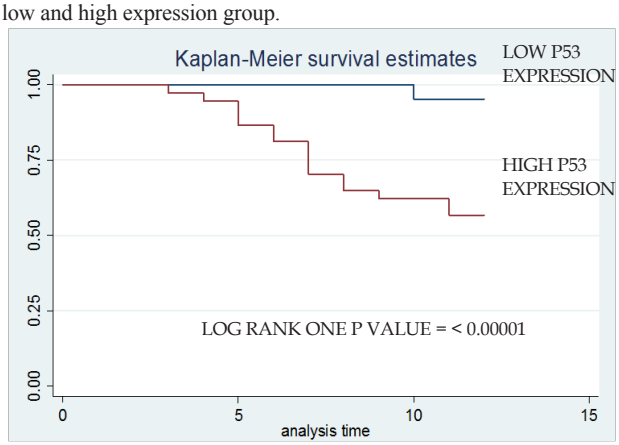
Tumorigenesis of Gastric cancer is a complex process which is affected by environmental as well as genetic factors. The exact pathogenesis of gastric cancer remains unclear; however various studies indicate it to be multifactorial.

The p53 is a tumor suppressor gene, localized to chromosome 17q13.1 and is classically considered as the "guardian of the genome". P53 protein is a product of p53 gene, composed of 393 amino acids, which functions in G1 phase of cell cycle arrest to allow the repair of DNA damage and to prevent the cell from

Table 1 Immunoreaction score (IRS)[23].

Table 1A Percentage positive cells		**Table 1B** Staining intensity	
percentage of p53 positive cells	Score	staining intensity	Score
≤ 10%	1	Negative	0
11-49%	2	Weak	1
50-79%	3	Moderate	2
≥ 80%	4	Strong	3
IRS score = Table 1A × Table 1B			
Total score = 0 to 12 {≤ 6 = low and > 6 = high}			

Chart: 1 Survival analysis using Kaplan meier method between p53 low and high expression group.



The Kaplan Meier survival curve of Gastric cancer patients (n=58). Patients having high levels of p53 protein expression are associated with a poor survival.

Table 2A Comparison of clinicopathological parameters with p53 expression.

Charactaristics	**Total**	**P53**		**P Value**
		Low Expression	**High Expression**	
Gender				
male	38	15	23	0.476
female	20	6	14	
age				
< 60	25	14	11	0.006*
≥ 60	33	7	26	
Tumour location				
proximal	18	2	16	0.008*
distal	40	19	21	
Tumour size				
≤ 5 cm	25	18	7	0.0001*
> 5 cm	33	3	30	
Pathological grade				
well diff.	17	13	4	0.0001*
mod diff.	21	6	15	
poorly diff.	20	2	18	
lauren classification				
intestinal	30	15	15	
diffuse	18	3	15	0.06
intermediate	10	3	7	
Histological type				
Tubular	28	13	15	
Pappilary	10	5	5	
Signet ring	10	3	7	0.09
Mucinous	5	0	5	
Mixed	5	0	5	
• * = Statistically significant				
• The χ^2 test was used to evaluate the association between p53 expression and clinicopathological parameters.				

Table 2B Comparison of TNM and Clinical stage with p53 expression.

Charactaristics	**Total**	**P53**		**P Value**
		Low Expression	**High Expression**	
T1	10	9	1	
T2	14	8	6	0.0001*
T3	21	3	18	
T4	13	1	12	
N STAGE				
N0	2	1	1	
N1	15	9	6	0.138
N2	32	9	23	
N3	9	2	7	
M STAGE				
M0	45	20	25	0.015*
M1	13	1	12	
Clinical stage				
I	8	6	2	
II	14	10	4	0.0001*
III	23	4	19	
IV	13	1	12	
• * = Statistically significant				
• TNM classification done according to AJCC. T: Tumor invasion; N: lymph node involvement, M=Metastasis.				
• The χ^2 test was used to evaluate the association between p53 expression and clinicopathological parameters.				

entering into the S phase or alternatively to guide damaged cells to apoptosis. So p53 played major role in cell cycle regulation, DNA repair and cell apoptosis. Mutation in p53 results in the loss of its ability to induce cell death leading to uncontrolled cell growth which promotes tumorigenesis. Normally p53 gene is not detected immunohistochemically but when mutated p53 becomes stabilized and has increased half life, thus it accumulates in the cell nucleus and can be detected immunohistochemically using monoclonal antibodies[24-27].

Mutations of the p53 gene have been observed in many malignancies and are found in ~30%-50% of lung, colorectal, head and neck, ovarian cancers and esophageal cancer and in ~5% of leukemia, sarcoma, melanoma, testicular cancer, and cervical cancer patients[28,29]. This lead to many observers to study p53 mutation profile meticulously in gastric cancer patients also. Laboratory analysis of p53 gene is done by three methods: (1) Polymerase chain reaction (PCR) (2) Detection of serum p53 antibody and (3) Immunohistochemistry (IHC)[30] In comparison to DNA sequencing, immunohistochemical methods are cheaper, easier, widely available throughout the world and more familiar to pathologists. P53 protein accumulation not only represent mutated p53 gene but also represent effect of other genes on its expression, so expression of p53 needs to be assessed separately for survival prediction.

P53 protein expression is found to be variable, may be because of using different antibody and different techniques of analysis by different studies. P53 expression is found in about 19% to 90% patients of gastric cancer. A study done by Akshatha C *et al*, Fenoglio-Preiser *et al*, Brito *et al* and Ghaffarzadegan *et al*[31-34] noted p53 positivity in 62.5 %, 19%, 35% and 75% of gastric carcinoma patients respectively. In our study we found 90% patients of gastric cancer showing p53 expression.

Daniela lazar et al in 2010[35] showed that there was insignificant association with gender, in spite of having higher incidence of gastric cancer in male patients. Similar results were seen in our study.

Risk of carcinogenesis increases with increase in age, a study done by Honda T *et al*[36] confirmed that age group of > 60 years has significantly higher risk for gastric cancer. Similar results were seen in our study i.e. p53 expression is significantly higher in age group of > 60 years. But these results are not confirmed by Daniela lazar *et al*[35].

One of the important parameter to assess prognosis in stomach cancer patients is histopathologic grade of tumor. As grade increases prognosis become poorer. When p53 expression was compared with histhopathological grading we found that its expression increases significantly with the increasing histopathological grades. So overexpression of p53 can be linked with histological aggressiveness of the tumor. Similar results were seen by Sasaki I *et al*[37] while some other studies like study done by Akshatha C *et al*[31] showed no correlation with histological grade.

The Lauren classification is frequently used in gastric cancer patients. It is based on how the gastric tissue looks and behaves when examined under a microscope. It divides adenocarcinoma of the stomach into 3 main types: intestinal, Diffuse and mixed type. Generally diffuse type behaves more aggressively. Intestinal type of gastric adenocarcinoma was the commonly observed type in our study which is similar to the observation noted by Nabi *et al* and Omran *et al* previously[38,39].

When p53 expression compared with lauren classification we found no significant correlation between these two parameter. Similar results were obtained by Pinto-de-Sousa J *et al*[40] and Akshatha C *et al*[31] but contradictory results also available as Daniela lazar *et al*[35] showed significant correlation between lauren classification and p53 expression.

Table 3 Univariate analysis to identify the factors that affect the survival.

Variables	Odds Ratio (Or)	Confidence Interval (Ci)	*P* Value
Age	2.2	0.60-9.7	0.175
Gender	0.46	0.124-1.77	0.194
Tumour location	1.9	0.48-7.24	0.282
Tumour size	9.5	1.7-93	0.0019*
HP greading	4.3	0.80-43	0.05*
HP type			0.465
Lauren classification	1.8	0.56-6.8	0.301
T stage	4.9	1.09-29.7	0.01*
N stage			0.354
M stage	10.4	2.1-55.7	0.0003*
Clinical stage	7.14	1.3-70.1	0.0082*
P53 expression	15	1.9-670	0.002*
• *: Statistically significant			
• *P* <0.05 was considered as a statistically significant difference.			
• OR: odds ratio ; CI: confidence interval.			

Table 4 Multivariate analysis to identify the factors that independently affect the survival

Variables	Odds Ratio (Or)	Confidence Interval (Ci)	P Value
Gender	2.64	0.58-11.96	0.206
N stage	0.366	0.102-1.31	0.123
M stage	0.224	0.045-1.10	0.066
P53 expression	9.34	1.003-90.9	0.049**
• **: Statistically significant			
• The Cox proportional hazards model was used to find out the factors that had a significant influence on overall survival			
• *P* <0.05 was considered as a statistically significant difference.			
• OR: odds ratio; CI: confidence interval.			

Location of the gastric tumor is important parameter to assess prognosis, as proximal gastric tumors behaves more aggressively than distal gastric tumors. So we analyzed p53 expression with tumor location we found significantly higher p53 expression in proximal tumors than distal tumors. Similar results were found by Fenoglio-Preiser CM *et al*[41].

Stomach cancer classified into various histological types according to WHO classification and when compared with p53 expression we found no correlation. Similar results were seen in studies done by Akshatha C *et al*, Daniela lazar *et al*[31,35].

Other important parameters are T, N, M & clinical stage. As the stage increases patients survival decreases. On comparison with these parameters, we found that p53 expression was significantly increased with increasing grades of T, M & clinical stages (I to IV) [Table 2]. Hence p53 expression can also be linked with invasiveness & clinical aggressiveness of the tumor.

Whereas our analysis also shows p53 expression was not correlated with N stage. Similar finding were seen in Akshatha C *et al*, Daniela lazar *et al* and Filiz *et al*[31,35,42].

The results of Kaplan meier analysis demonstrates that patients with high p53 expression show significantly poor survival than the patients with low p53 expression. This result contradicts many previous studies, which fails to show association between p53 expression & survival[15-17]. The results of univariate analysis showed that tumor size, histopathological grading, T stage, M stage, clinical

stage and p53 expression were significantly correlated with the survival. Additionally, multivariate analysis revealed p53 expression was found to be independent variable affecting gastric cancer patient's survival.

CONCLUSION

Significant numbers of gastric cancer patients demonstrated increased expression of p53 and is found to be independent variable affecting survival. Finally we arrived at conclusion; Immunohistochemical analysis of p53 is simple & effective modality which can be used to determine the prognosis and survival in various grades & stages of gastric cancer.

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Peer reviewer: Jorge Serra Colina