Original Article

Yield of p53 expression in esophageal squamous cell cancer and its relationship with survival

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Abstract

Background/Aims: Esophageal squamous cell carcinoma (ESCC) is the most aggressive type of cancer. Mutation of tumor suppressor gene p53 is observed in many gastrointestinal malignancies including ESCC. The immunohistochemical protein expression of mutant p53 has been proposed as a potential tool to evaluate the biological behavior of ESCC. Predictive value of p53 for survival is debatable, hence this study was formulated to know the survival of patients with p53 expression in ESCC.

Patients and Methods: We prospectively included 91 consecutive patients of ESCC from August 2014 to August 2016. Biopsy specimens were treated immunohistochemically and expression of p53 gene was analyzed by Immunoreactive Score (IRS). These findings were then compared with clinicopathological parameters such as age, gender, histological grades, and TNM stages. All patients received treatment and were kept under regular follow-up.

Results: M: F ratio was 2.03:1. p53 expression analyzed by IRS showed low expression (score ≤6) in 35 patients (38.46%) and high expression (>6) in 56 patients (61.54%). Level of p53 expression increased significantly with increasing histological grades of ESCC and TNM stage ($P \leq 0.001$). Multivariate analysis shows p53 expression as independent predictor of survival. After 1 year of follow up, survival in the p53 high-expression group was 67.86% [standard error (SE) = 0.0473, confidence interval (CI) = 0.75–0.97) and in low p53 expression group was 91.43% (SE = 0.06, CI = 0.53–0.78) with statistically significant difference $P = 0.0001$ when analyzed with Kaplan–Meier method.

Conclusion: Expression of p53 correlates with the survival and is a simple, effective and reproducible modality to determine the prognosis and survival in ESCC.

Keywords: Esophageal cancer, gene expression, immunohistochemistry

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INTRODUCTION

Esophageal carcinoma is one of the most aggressive type of cancer worldwide. It is the sixth frequent cause of cancer associated mortality in the world.[1] In India, the highest incidence of esophageal carcinoma has been reported from Kashmir valley and north-east states.[2,3]

Esophageal cancer is classified into two major histological subtypes, namely squamous cell carcinoma and adenocarcinoma. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype. Prognosis of ESCC is poor as patients generally consulted health care services in an advanced stage of disease. Prognosis and survival depends on early diagnosis and treatment.

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However, specific patients with the same stage have different prognosis. So there is constant search for specific histological and biological markers in order to identify the subgroups of patients with more aggressive course of disease within the same stage of illness. Many molecular alterations occur in esophageal carcinogenesis[4] and further investigation into these protein alterations may provide clues to discover novel markers for improving diagnosis and guiding-targeted therapy.[5] Many such potential markers were studied, viz. Ki67, hypoxia inducible factor, E-cadherin, matrix metalloproteinase-1, transforming growth factor-B in ESCC, but among all, p53 was studied considerably.[6,7]

Cancer is an abnormal growth of cells which might be due to a defect in cell cycle regulation. Cell cycle checkpoints regulate the cell cycle and maintain the genomic integrity of the cells during DNA replication. Deregulation of the cell cycle leads to tumorigenesis. Genetic and epigenetic changes affecting cell cycle regulating genes are the major events during carcinogenesis. p53 (p14/MDM2/p53) pathway is one of the major cell cycle pathways involved in cell cycle regulation. Genetic and epigenetic alterations in this pathway lead to inactivation of these genes, thus leading to uncontrolled proliferation of damaged DNA, which turns into cancer formation. p53 pathway is frequently found to be mutated in ESCC. It is well known that these mutations can lead to an increase in expression of p53, which accumulates in nuclei and can be detected by immunohistochemistry (IHC) methods.[8]

The immunohistochemical protein expression of p53 has been proposed as a potential tool to evaluate the behavior biologically. Majority of studies suggest a prognostic significance of p53 expression in esophageal cancer, however, controversial results do exist.[9-11] These conflicting results led us to formulate the study for assessing the yield of p53 expression in ESCC and its relationship with survival.

PATIENTS AND METHODS

Patients

This was a prospective study carried out at the Department of Gastroenterology, Government Medical College and Super Speciality Hospital, Nagpur, India. The study protocol was approved by our Institutional Ethics Committee (approval no: 762/EC/Pharmac/GMC/NGP). The study includes 91 consecutive patients of squamous cell esophageal carcinoma who were diagnosed by the use of endoscopy [Figure 1a] and histopathology [Figure 1b] from August 2014 to August 2015. Patients were followed

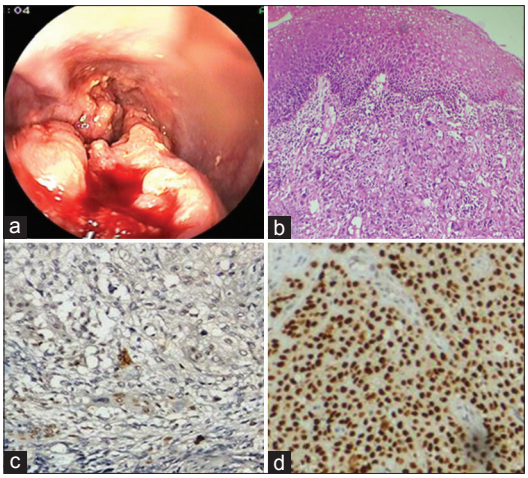

Figure 1: (a) Endoscopy showing esophageal malignancy; (b) Histopathology of ESCC; (c) ESCC with low expression of p53; (d) ESCC with high expression of p53

prospectively for 12 months (or till August 2016) and/or death from the date of enrollment. The overall survival in our study was defined as duration of survival to 12 months and/or death.

After fixation in formalin biopsy, tissue specimens were embedded in paraffin and were sent for IHC. The following parameters were evaluated: age, gender, tumor location, tumor size, TNM staging [according to American Joint Committee on Cancer (AJCC)][12] using computed tomography scan of thorax and upper abdomen, and histopathological grading according to World Health Organization classification.[13] All patients received standard of care treatment according to the stage of disease.

Immunohistochemistry

IHC 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 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval and then bench cooled for 20 min and the same cycle was repeated again. To prevent nonspecific reactions, sections were incubated with 10% serum for 10 min. Prediluted 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 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 (DAB) (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.[14]

The IHC staining of mutant (MT) p53 was assessed according to the Immunoreactive Score (IRS) [Table 1a and b], 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 intranuclear DAB staining (brown color) [Figure 1c and d]. The percentage of positive cells was assessed with the help of labeling index (p53 Labeling index = Number of IHC positive cells × 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.

Immunoreaction score (IRS) is calculated as follows: [15]

IRS score = Table 1a × Table 1b

Total score = 0 to 12 {≤6 = low and >6= high}

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 these factors and overall survival. A *P* value <0.05 was considered as statistically significant.

RESULTS

Totally 91 patients of ESCC were enrolled in our study. Among them, 61 (67%) patients were males and 30 (33%) were females with a M: F ratio of 2.03:1. Age range in the study population was 35–80 years with the mean age of 58.5 (SD ± 9.67) years.

Expression profile of p53 in ESCC patients

Mp53 expression was observed in 90% of ESCC patients. Among 91 patients, 90% of patients showed positive Mp53 expression and the remaining 10% of patients showed 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. Thus, the low-expression group patients were 35 (38.46%) and high-expression group patients were 56 (61.64%).

Table 1a: Percentage positive cells

Percentage of p53 positive cells	Score
≤10%	1
11-49%	2
50-79%	3
≥80%	4

Table 1b: Staining intensity

Staining intensity	Score
Negative	0
Weak	1
Moderate	2
Strong	3

Correlation of p53 expression profile with different clinicopathological features

Level of p53 expression was found significantly higher in patients with age group ≥60 years than <60 years ($P = 0.023$). Similarly, higher p53 expression was found in patients who are betel nut chewers ($P \leq 0.0001$). p53 expression was found significantly augmented with higher histological grade of cancer ($P \leq 0.001$). Significant correlation was found between p53 expression and higher T stage ($P = 0.005$), N stage ($P = 0.001$), and M stage ($P = 0.012$). As clinical stage of cancer increases, increased expression of p53 was present and has significantly correlated with it ($P = 0.004$). But no statistically significant correlation was found between gender and p53 expression profile ($P = 0.0481$) [Table 2].

Correlation of p53 expression profile with survival outcome

On Kaplan–Meier survival analysis, patients with p53 high expression had significantly shorter overall survival than those patients with low p53 expression (log-rank $P < 0.0001$) [Chart 1]. At 12th month of follow-up, 67.89% (CI 0.75–0.97) of patients with p53 high-expression group survived and 91.43% (CI 0.53–0.76) of patients with p53 low-expression group survived.

Multivariate analysis of correlation of p53 expression profile with overall survival

Multivariate analysis by Cox regression model further shows that high p53 expression was an independent predictor of overall poorer survival [heart rate (HR) = 5.5; 95% CI 4.1–13.51, $P = 0.005$]. It also showed that increasing age (HR = 9.7; 95% CI 1.2–79, $P < 0.03$),

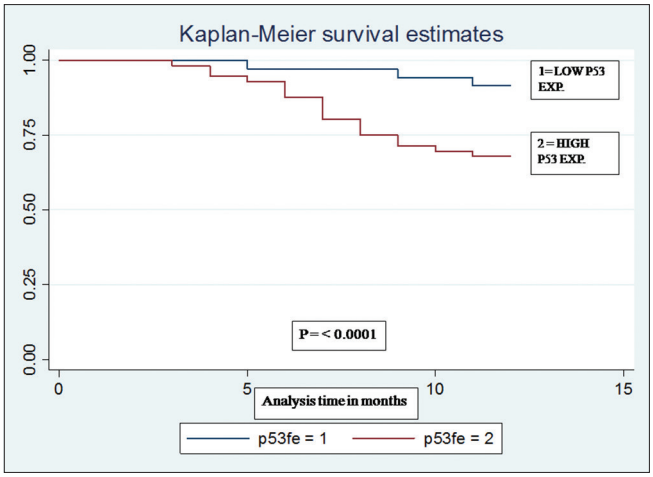

Chart 1: The Kaplan–Meier survival curve for ESCC patients ($n = 91$). According to p53 protein expression level, ESCC patients exhibiting high levels of p53 protein expression are associated with a poor survival

higher M stage (HR = 0.001; 95% CI 1.4–0.02, $P < 0.003$), and higher clinical stage (HR = 14.16; 95% CI 3.5–5.6, $P < 0.001$) were found to be important predictors of poorer overall survival. However, gender, histological grade, T stage, N stage, and tumor size were not significant predictors of survival in ESCC patients [Table 3].

DISCUSSION

Tumorigenesis of ESCC is a complex process which is affected by environmental as well as genetic factors.[16] The exact pathogenesis of ESCC 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 entering into the S phase of the cell cycle or alternatively to guide the damaged cells to apoptosis. So p53 plays a 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 an increased half-life; thus it accumulates in the cell nucleus and can be detected immunohistochemically using monoclonal antibodies.[17-20]

Mutations of p53 gene have been observed in many malignancies and are found in ~30–50% of lung, colorectal, head and neck, and ovarian cancers, and in ~5% of leukemia, sarcoma, melanoma, testicular cancer, and cervical cancer patients.[21,22] This led many observers to study p53 mutation profile meticulously in esophageal

Table 2: Comparison of clinicopathological parameters with p53 expression

Characteristics	Total	p53		*P*
		Low expression	High expression	
Gender				
Male	61 (67%)	25	36	<0.481
Female	30 (33%)	10	20	
Age				
<60	41 (45.05%)	21	20	<0.023*
≥60	50 (54.95%)	14	36	
Betel nut chewing				
Yes	61 (67.03%)	7	54	<0.0001*
No	30 (32.97%)	28	2	
Pathological grade				
Well diff.	30 (33%)	21	9	<0.001*
Moderately diff.	39 (42.9%)	13	26	
Poorly diff.	22 (24.2%)	1	21	
TNM and Clinical Stage				
T1	7 (7.7%)	5	2	<0.005*
T2	17 (18.7%)	11	6	
T3	22 (24.2%)	9	13	
T4	45 (49.5%)	10	35	
N Stage				
N0	14 (15.4%)	10	4	<0.001*
N1	31 (34.1%)	16	15	
N2	39 (42.9%)	8	31	
N3	7 (7.7%)	1	6	
M Stage				
M0	74 (81.3%)	33	41	<0.012*
M1	17 (18.7%)	2	15	
Clinical stage				
I	9 (9.9%)	7	2	<0.004*
II	18 (19.8%)	10	8	
III	47 (51.6%)	16	31	
IV	17 (18.7%)	2	15	

*: Statistically significant; The χ^2-test was used to evaluate the association between p53 expression and clinicopathological parameters. TNM classification was done according to AJCC. T: Tumor invasion; N: lymph node involvement; M: Metastasis

Table 3: Univariate analysis and multivariate analysis identify the factors that independently affect the survival

Variables	Univariate			Multivariate		
	RR	95% CI	*P*	RR	95% CI	*P*
p53 expression	7.4	6-9.1	<0.009	5.5	4.1-13.51	<0.005**
Age	4.8	1.5-15.14	<0.001	9.7	1.2-79.56	<0.03**
Gender	0.98	0.44-2.17	<0.968	1.2	0.21-6.5	<0.831
Pathological grade	9.8	1.38-69	<0.004	1.8	0.51-6.7	<0.339
T Stage	-	-	<0.001	6.6	0.71-61.37	<0.097
N Stage	-	-	<0.0001	2.4	0.49-12.54	<0.266
M Stage	8.7	4.1-18.21	<0.0001	0.0001	1.4-0.02	<0.003**
Clinical stage	-	-	<0.0001	14.16	3.5-5.6	<0.001**
Tumor size	3.4	1.3-8.5	<0.004	0.84	0.12-6.1	<0.893

**: 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. RR: Relative risk; CI: Confidence interval

cancer patients also. Laboratory analysis of p53 gene was done by three methods: (1) polymerase chain reaction; (2) detection of serum p53 antibody; and (3) IHC. In comparison to DNA sequencing, immunohistochemical methods are cheaper, easier, widely available throughout the world, and more familiar to pathologists as a standard procedure. p53 protein accumulation not only represents mutated p53 gene but also represents effect of other genes on its expression, so expression of p53 needs to be assessed separately for survival prediction.

p53 protein expression is variable may be because of using different antibody and different techniques of analysis by different studies. p53 expression is found in about 50–90% patients of ESCC. Previous studies showed 41-87% of p53 positivity in ESCC patients.[23-26] In our study, we found 90% patients of ESCC showing p53 expression.

Yao *et al*.[27] in 2014 showed that there was insignificant association with gender, in spite of having higher incidence of ESCC in male patients. Similar results were seen in our study. Risk of carcinogenesis increases with increase in age. A study done by Cummings *et al*. confirmed that age group of >60 years has significantly higher risk for ESCC.[28] Similar results were reflected in our study that p53 expression is significantly higher in age group of >60 years.

Areca nut chewing was significantly and independently associated with an increased risk of ESCC in Asians.[29] If fermented areca nut is used or it is used in combination with tobacco, risk for ESCC increases by many fold. When p53 expression was compared with betel nut chewing, we found that p53 expression was significantly high in patients with a habit of betel nut chewing. Similar results were reported by Goan *et al*.[30]

One of the important parameters to assess prognosis in ESCC patients is histopathologic grade of tumor. As grade increases, prognosis becomes poorer. When p53 expression was compared with histopathological grading, we found that p53 expression was significantly increased with increasing grades of histopathology. Similar results were seen by Huang *et al*. in 2014.[31] Hence overexpression of p53 can be linked with histological aggressiveness of the tumor. Similarly other important parameters are T, N, M, and clinical stage. As the stage increases, patients' survival decreases. On comparing these parameters, we found that p53 expression was significantly increased with increasing grades of T, N, M, and clinical stages (I to IV) [Table 1]. Similar results were seen by Kate Haung *et al*.[31] Hence p53 expression can also be linked with invasiveness and clinical aggressiveness of the tumor.

In our study Kaplan–Meier analysis demonstrated that patients with high p53 expression show significantly poor survival than patients with low p53 expression. This result concurs with the observations of Yao *et al*.[27] but also contradicts many previous studies, which failed to show association between p53 expression and survival.[9-11] The results of univariate analysis showed that age, pathological grade, tumor size, T stage, N stage, M stage, and TNM clinical stage were significantly correlated with poor prognosis. Additionally, multivariate analysis revealed the age, M stage, clinical stage, and p53 expression were found to be independent variables affecting ESCC patient survival.

CONCLUSION

In conclusion, a significant number of ESCC patients had increased expression of p53, and it significantly correlates with age, betel nut chewing, histological grade, TNM, and clinical stage. p53 was an independent variable affecting the survival. Immunohistochemical analysis of p53 is simple, effective, and reproducible modality that can be used to determine the prognosis and survival in various grades and stages of ESCC.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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