Original Article

Expression of epidermal growth factor receptor, p53, Bcl2, vascular endothelial growth factor, cyclooxygenase-2, cyclin D1, human epidermal receptor-2 and Ki-67: Association with clinicopathological profiles and outcomes in gallbladder carcinoma

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Abstract

Background: The present study observed the expression levels of epidermal growth factor receptor (EGFR), p53, Bcl2, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (cox-2), cyclin D1, human epidermal receptor-2 (HER-2) and Ki-67 in gallbladder carcinoma (GBC) and their association with clinicopathological profiles and disease outcomes. Materials and Methods: Fifty consecutive samples of cholecystectomy/biopsies from GB bed (archived formalin fixed paraffin embedded tissue blocks of different stages of GBC) were included, and patient details related to their demographic profile, investigations, tumor profile, treatment, and follow-up were recorded. Immunohistochemistry was performed to study the expression levels. Results: Overexpression of EGFR, p53, Bcl2, VEGF, cox-2, cyclin D1 and HER-2 was observed as 74%, 44%, 8%, 34%, 66%, 64%, and 4%, respectively. Association of Bcl2 overexpression in mucinous morphology (40%, \( P = 0.045 \)), cox-2 overexpression in early stage (I/II) tumors (87.5%, \( P = 0.028 \)) and VEGF overexpression in alive patients (47.1%, \( P = 0.044 \)) was observed. Co-expression of EGFR and p53 were statistically significant (\( P = 0.033 \)). Ki-67 labeling index was significantly higher in patients in age group <40 years (\( P = 0.027 \)), and poorly differentiated tumors (\( P = 0.023 \)). Advanced disease and poorly differentiated tumors showed a significantly poor median survival (\( P < 0.05 \)). Conclusion: EGFR, cox-2 and cyclin D1 were largely overexpressed. Advanced tumor stages and poorly differentiated tumors are predictors of poor survival.

Keywords: Cyclin D1, epidermal growth factor receptor, gallbladder carcinoma, human epidermal receptor-2, p53, vascular endothelial growth factor

INTRODUCTION

Gallbladder carcinoma (GBC) is the most common cancer of the biliary tract with a particularly high incidence in Chile, Japan, and Northern India.\(^1\)\(^,\)\(^2\) It accounts for 17,262 new cases and 10279 deaths in India.\(^3\) Worldwide, Delhi has the highest incidence rate (21.5/100,000) for GBC in
females. GBC is the cause of 11.4% of total cancer deaths in females in Delhi. The prevalence of GBC is also common in the gangetic belt of Northern India. The prognosis is dismal, and survival is poor for patients who present with unresectable disease. However, curative resection of the tumor provides cure and long-term survival benefits. This cancer is difficult to diagnose and usually fatal because of late clinical presentation and lack of effective treatment modalities. Onset of the disease is accompanied by multiple genetic changes that result in altered gene expression, which lead to the development of carcinoma.

Gallbladder carcinoma has been enigmatic in terms of molecular progression and expression of various biomarkers. This lack of information stems possibly from advanced disease, short median survival, and relatively low incidence of disease in most parts of the world. ErbB family of receptors has been known to play a key role in carcinogenesis and disease progression in different types of cancer. Epidermal growth factor receptor (EGFR) and human epidermal receptor-2 (HER-2) are transmembrane receptor protein kinase and members of ErbB family. Ligand binding to the extracellular domain of the receptor causes protein kinase activation in the form of downstream signaling that led to the growth, proliferation, invasion and metastasis of the cell. Vascular endothelial growth factor (VEGF) is a key element in the initiation of tumor angiogenesis that promotes the tumor cell survival and metastasis. The overexpression of VEGF has been found to be a prognostic factor in different cancers. The tumor suppressor gene P53 functions as a promoter of apoptosis, and it is one of the most frequent genetic alterations in a variety of cancers. Abnormal expression of the anti-apoptotic protein Bcl2 can modify the biological behavior and progression of cancer. Overexpression has also been reported in different cancers. Loss of cell cycle regulatory control contributes to the uncontrolled division and proliferation of cell. Cyclin D1 is an important protein that regulates the cell cycle by exerting an effect on cyclin-dependent kinases that allow cells to enter into S phase. Overexpression of cyclin D1 has been reported in different cancers. Cyclooxygenase-2 (cox-2) protein acts as a pro-inflammatory mediator in the process of chronic inflammation that drives the cancer initiation. Elevated expression of cox-2 has been observed in gastric and colon cancers and associated with invasion and metastasis. Ki-67 is a proliferative marker which usually expresses in all active phases of the cell cycle. This antigen can be detected in the nucleus of tumor cell indicating its proliferative activity. High labeling index (LI) predicts early recurrence after surgery in tumors.

There are variations in the data reported across the literature for the biomarkers in the study. Most studies are confined with cholangiocarcinoma or biliary tract cancer. So far, not much work has been done in relation with these markers in GBC and scanty data is available from India on the expression of these biomarkers in GBC. Thus, the present study was conducted to observe the expression levels of EGFR, p53, Bcl2, VEGF, cox-2, cyclin D1, HER-2 and Ki-67 in GBC and to find their association with the clinicopathological profiles and the disease outcome.

**MATERIALS AND METHODS**

Fifty consecutive cholecystectomy or biopsy samples in the form of archived formalin fixed paraffin embedded tissue blocks of different stages of GBC resected at Rajiv Gandhi Cancer Institute and Research Center during the year 2008-2011 have been included in the study retrospectively. The tumor histology of the selected cases were adenocarcinoma (86%) and adenosquamous carcinoma (14%). Details of each patient related to their demographic profile, investigations, tumor profile, including metastasis and histopathology details, further treatment and follow-up information was also recorded. Survival status of 46/50 patients was obtained from the medical records as well as by telephonic calls, rest were not traceable and hence, not evaluated for survival analysis. The Institutional Review Board of Rajiv Gandhi Cancer Institute and Research Center had granted a waiver for this retrospective study. All the markers have been reported as per the reporting recommendations for tumor marker prognostic studies criteria.

Immunohistochemistry (IHC) was performed on the archived block with adequate tumor to study the expression of EGFR, p53, Bcl2, VEGF, cox-2, cyclin D1 and Ki-67 markers. Serial sections of 5 μm were cut from the selected representative blocks with tumor. Rabbit anti-human monoclonal antibody to EGFR (Clone SP4, Diagnostics Biosystems, USA), cyclin D1 (SP4, Biocare Medical, USA), cox-2 (Clone SP21, Biocare Medical, USA), and mouse anti-human monoclonal antibodies to human VEGF (Clone VG1, DakoCytomation, Denmark), Bcl2 (Clone 124, Dako, Denmark), p53 (DO-7, Novacastra Laboratories), Ki-67 antigen (Clone MIB-1, Dako, Denmark) were used. IHC was performed using Envision Kits (Dako, Denmark) in accordance with the manufacturer’s instructions. For HER-2 expression, HercepTest kit (Dako, Denmark) was used.

Positive staining was defined as weak (>10-<25% of positive cells), moderate (25-75% of positive cells) and strong (>75% of positive cells) whereas intensity of staining was considered to be the week, moderate, and strong. Final scores of the IHC were marked based on the percentage of positive cells and intensity of staining, and classified as negative, weak, moderate and strong overexpression. Breast cancer scoring was followed for the scoring of HER-2 staining. HER-2 expression was interpreted as negative for protein index.
expression 0 and 1, weakly positive for 2+ staining and strong positive for 3+ staining.

Statistical Package for the Social Sciences (SPSS) version 16 for Windows (SPSS Inc., Chicago IL, USA) was used for all the statistical analysis. Pearson \( \chi^2 \) or Fisher’s Exact Test, whichever was appropriate, was applied for categorical variables. Mann–Whitney U-test was used to test the association between categorical and continuous variables. For the survival analysis, Kaplan–Meier method\(^\text{23}\) was applied, and Log Rank test was used to compare the difference in survival among the groups. A two sided \( P < 0.05 \) was considered as significant.

**RESULTS**

A total of 50 consecutive cases of GBC in the period 2008–2012 were included in this study. Median age of the patients was 52 years (range: 23–78 years), and among them 80% cases were females. Most of the tumors were moderately differentiated (68%). Tubular morphology was the most common type in about 50% cases. Sixty-eight percent of cases presented with advanced stages of the disease (III/IV), according to the classification system by American Joint Committee on Cancer Staging 2010.\(^\text{24}\) Nodal metastasis was seen in 42% and metastasis to the distant organ was observed in 14% of cases. Overexpression of EGFR, p53, Bcl2, VEGF, cox-2, cyclin D1, HER-2 was observed as 74%, 44%, 8%, 34%, 66%, 64% and 4%, respectively [Figure 1]. The overexpression of EGFR, p53, cyclin D1 and HER-2 were not correlated with any of the clinicopathological factors [Table 1]. In an age <40 years, EGFR, Bcl2, VEGF, cox-2, cyclin D1 were overexpressed, while p53 and HER-2 were overexpressed in the age group >40 years. Bcl2, VEGF, cox-2 and cyclin D1 positivity was observed more in males, whereas EGFR p53 and HER-2 in females. In advanced stages of disease p53, Bcl2 cyclin D1 and HER-2 were strongly expressed in comparison to EGFR, VEGF, cox-2 that presented in the early stages. Increased positivity of EGFR, cox-2 and cyclin D1 were observed in the tumors with tubular morphology. In papillary tumors, higher expression of EGFR, VEGF, cox-2 and HER-2 was seen, however, in mucinous tumors, Bcl2 was found to be overexpressed. EGFR and cox-2 were highly expressed in the well-differentiated tumors, while p53, VEGF and HER-2 in well-differentiated tumors. In poorly differentiated tumors, increased positivity of EGFR, p53, Bcl2 and cyclin D1 was observed. Stronger expression of p53, Bcl2, cyclin D1 and HER-2 was observed in tumors with nodal and/or distant metastasis. Overall, Bcl2 overexpression in mucinous morphology (40%, \( P = 0.045 \)), cox-2 overexpression in early stage (II/III) tumors (87.5%, \( P = 0.028 \)) and VEGF overexpression in the alive patients (47.1%, \( P = 0.044 \)) were associated significantly. It was noted that all the well-differentiated tumors (3/3) were positive for EGFR and cox-2 overexpression, although, the result was not statistically significant.

Overexpression of markers has not shown any significant comparison of positive or negative expression of one biomarker with others provided a significant combined effect of EGFR with p53 (\( P = 0.033 \)). Co-expression of EGFR with cox-2, EGFR with cyclin D1, and Cox2 with cyclin D1 was observed in 26 (52%), 24 (48%) and 20 (40%) cases, respectively; however, the result did not attain statistical significance [Table 2].

Ki-67 LI was significantly higher in patients in the age group <40 years (40.0 ± 11.5; \( P = 0.027 \)), and poorly differentiated tumors (36.2 ± 9.6; \( P = 0.023 \)). Other clinicopathological factors and expression profile of markers did not show any correlation with Ki-67 LI [Table 3].

Median follow-up time was 15.5 months, and 29 deaths were observed in the study [Table 1]. Median overall survival was 21 months (95% confidence interval 14.947–27.053). Overexpression of markers has not shown any significant
Table 1: Correlation of overexpression of biomarkers with clinicopathological characteristics (n=50)

| Variables                      | n  | EGFR  | p53  | Bcl2  | VEGF  | Cox-2 | Cyclin D1 | HER-2 |
|--------------------------------|----|-------|------|-------|-------|-------|-----------|-------|
|                                | n (%) | P     | n (%) | P     | n (%) | P     | n (%)     | P     |
| Age                            |     |       |      |       |       |       |           |       |
| <40                            | 7    | 0.168 | 2.86 | 0.444 | 1.43  | 0.609 | 5.17      | 0.210 |
| >40                            | 43   | 69.8  | 20   | 46.5  | 3.70  | 10.0  | 13.2      | 2.62  |
| Sex                            |     |       |      |       |       |       |           |       |
| Male                           | 10   | 7.00  | 0.707| 4.40  | 0.00  | 1.00  | 4.00      | 0.717 |
| Female                         | 40   | 75.0  | 18   | 45.0  | 3.75  | 13.25 | 20.0      | 6.20  |
| Stage                          |     |       |      |       |       |       |           |       |
| I-II                           | 16   | 93.8  | 0.39 | 6.37  | 0.525| 0.00  | 7.43      | 0.318 |
| III-IV                         | 34   | 64.7  | 16   | 47.1  | 4.11  | 10.0  | 29.4      | 5.59  |
| Tumor morphology               |     |       |      |       |       |       |           |       |
| Tubular                        |     |       |      |       |       |       |           |       |
| No                             | 25   | 68.0  | 0.333| 13   | 52.0  | 0.254| 12.0      | 0.609 |
| Yes                            | 25   | 80.0  | 0.360| 0    | 4.0   | 1    | 28.0      | 1.87  |
| Papillary                      |     |       |      |       |       |       |           |       |
| No                             | 43   | 72.1  | 0.660| 19   | 44.2  | 1.00  | 4.93      | 1.00  |
| Yes                            | 7    | 85.7  | 3    | 42.9  | 0    | 0    | 42.9      | 71.4  |
| Mucinous                       |     |       |      |       |       |       |           |       |
| No                             | 45   | 75.6  | 0.595| 21   | 46.7  | 0.368| 4.40      | 0.045 |
| Yes                            | 5    | 60.0  | 1    | 20.0  | 2    | 44.0 | 20.0      | 3.00  |
| Tumor differentiation          |     |       |      |       |       |       |           |       |
| Well                           |     |       |      |       |       |       |           |       |
| No                             | 47   | 72.3  | 0.558| 22   | 46.8  | 0.246| 4.83      | 1.00  |
| Yes                            | 3    | 100.0 | 0    | 0    | 0    | 0    | 133.3     | 3.00  |
| Moderate                       |     |       |      |       |       |       |           |       |
| No                             | 16   | 87.5  | 0.179| 7    | 43.8  | 1.00  | 12.5      | 0.584 |
| Yes                            | 34   | 67.6  | 15   | 44.1  | 5    | 5.9  | 38.2      | 22.0  |
| Poor                           |     |       |      |       |       |       |           |       |
| No                             | 37   | 70.3  | 0.469| 15   | 40.5  | 0.406| 2.55      | 0.275 |
| Yes                            | 13   | 84.6  | 7    | 53.8  | 2    | 15.4 | 23.1      | 8    |
| Nodal metastasis               |     |       |      |       |       |       |           |       |
| No                             | 29   | 79.3  | 0.314| 14   | 48.3  | 0.474| 2.69      | 1.00  |
| Yes                            | 21   | 66.7  | 8    | 38.1  | 2    | 9.5  | 28.6      | 11.0  |
| Distant metastasis             |     |       |      |       |       |       |           |       |
| No                             | 43   | 76.7  | 0.357| 18   | 41.9  | 0.684| 2.47      | 0.089 |
| Yes                            | 7    | 57.1  | 4    | 57.1  | 2    | 28.6 | 28.6      | 42.9  |
| Status*                        |     |       |      |       |       |       |           |       |
| Alive                          | 17   | 76.5  | 1.000| 5    | 29.4  | 0.090| 1.59      | 1.00  |
| Dead                           | 29   | 75.9  | 16   | 55.2  | 2    | 6.9  | 17.2      | 18.6  |
| Ki-67 Li Positive              |     |       |      |       |       |       |           |       |
| Mean                           | 29.6 | 0.604 | 28.6 | 0.905 | 30.5 | 0.274| 30.6      | 0.594 |
| SD                             | 14.0 | 11.8  | 5.6  | 15.5  | 15.4 | 15.0 | 14.5      | 13.6  |
| Negative                       |     |       |      |       |       |       |           |       |
| Mean                           | 28.9 | 30.0  | 28.9 | 28.8  | 27.7 | 29.4 | 29.2      |       |
| SD                             | 17.2 | 16.9  | 15.2 | 14.5  | 13.6 | 14.7 | 14.7      |       |

\*n=46, P<0.05 was considered as significant. LI: Labeling index, SD: Standard deviation, EGFR: Epidermal growth factor receptor, VEGF: Vascular endothelial growth factor, HER-2: Human epidermal receptor-2, Cox-2: Cyclooxygenase-2

effect on the survival of patients. However, better survival was noted in EGFR negative, p53 negative, cyclin D1 negative, HER-2 negative, Bcl2 positive, VEGF positive and Cox-2 positive cases [Figure 2]. Among clinicopathological characteristics, advanced disease and poorly differentiated tumors have shown a significantly (P < 0.05) poor median survival [Figure 2]. Age, sex, tumor morphology and metastasis did not affect the survival significantly [Table 3].

**DISCUSSION**

Gallbladder cancer is a rare malignancy with variations in terms of geography, ethnicity and gender around the world. It frequently occurs in the northern part of India specially regions alongside the Gangetic belt.[2] The reasons of high prevalence in Gangetic belt were the presence of heavy metals that is, nickel, chromium
and cadmium in the water and presence of high levels of dichlorodiphenyltrichloroethane, an organochloride pesticide, in the soil.[25] GBC is one of most lethal cancers and more prevalent in females.[5] Carcinogenesis is a multistep process that involves mutations in different types of genes that may lead to the overexpression in different proteins. The present study aimed to determine the expression of various IHC based biomarkers and its correlation with various clinicopathological factors. To the best of our knowledge, this is the first study from India to analyze the role of EGFR, p53, Bcl2, VEGF, Cox-2, cyclin D1, HER-2, and Ki-67, and their correlation with clinicopathological factors and disease outcome.

In the present study, the most frequently observed clinicopathological characteristics were late age of disease presentation (age >40 years, 86%), female sex (80%), moderately differentiated adenocarcinoma (68%), tubular morphology (50%), advanced stages of the disease (68%), and extensive loco-regional and nodal metastasis (42%). The data is consistent with the published reports.[26-29] Advanced nature of the disease may be due to the late arrival of symptoms and late detection. Aggressive form results in the rapidly spreading of the tumor in the regional lymphnodes and adjacent organs like liver that makes it unresectable. Lack of awareness and early detection strategies of this disease in the community makes it difficult to diagnose at an early stage.

There are poor knowledge and understanding of the molecular pathogenesis of the disease. The biomarkers in the present study belong to some of the broad categories of molecules such as growth factor and receptors (EGFR, VEGF, HER-2), tumor suppressor or promoter of apoptosis (p53), antiapoptotic (Bcl2), cell cycle regulatory molecules (cyclin D1), enzyme implicated in the formation of prostaglandin (cox-2), and marker of cell proliferative activity (Ki-67). The overexpression of these molecules has shown implications in GBC and other cancers as well. In this study EGFR, cox-2 and cyclin D1 overexpression was detected in more than 50% of patients while Bcl2 and HER-2 were positive only in a small number of cases (8% and 4%, respectively). The overexpression of EGFR in GBC across the literature has been reported from 9.3% to 12.4% of cases.[18,26,30] It was associated with tumor progression and poor survival.[27,31] Other studies have also reported an increased expression of EGFR ranging from 38.5% to 93.7%,[32,33] however, the sample sizes of GBC is small in these reports. Overexpression of EGFR indicates that tumors of gallbladder could arise from the defects in the cell signaling molecules that affect the tyrosine kinase and other intracellular secondary messenger which ultimately exert its effect on the genes expression by the activation of transcription factors. In the present study it has also been noted that all the well-differentiated tumors (3 of 3) were positive for EGFR. However, no hypothesis about this finding may be proposed due to the small number of patients in this group.

### Table 2: Overexpression in one biomarker in the presence or absence of others (n=50)

| None/either/both | EGFR | p53 | Bcl2 | VEGF | Cox-2 | Cyclin D1 | HER-2 |
|------------------|------|-----|------|------|-------|-----------|-------|
| P value          |      |     |      |      |       |           |       |
| EGFR             | -    | 4/33/13 | 12/35/3 | 9/28/13 | 6/18/26 | 5/21/24 | 13/35/2 |
| P value          | 0.033 | 1.00  | 1.00  | 0.322 | 1.00  | 1.00     | 1.00  |
| p53              | 4/33/13 | -   | 24/26/0 | 16/29/5 | 7/31/12 | 11/24/15 | 27/22/1 |
| P value          | 0.033 | 0.121 | 0.136 | 0.130 | 0.585 | 1.00      |       |
| Bcl2             | 12/35/3 | 24/26/0 | -    | 31/17/2 | 17/29/4 | 17/30/3 | 44/6/0 |
| P value          | 1.00  | 0.121 | 0.597 | 0.285 | 1.00  | 1.00      |       |
| VEGF             | 9/28/13 | 16/29/5 | 31/17/2 | -    | 13/24/13 | 11/29/10 | 32/17/1 |
| P value          | 1.00  | 0.136 | 0.597 | 0.262 | 0.584 | 1.00      |       |
| Cox-2            | 6/18/26 | 7/31/12 | 17/29/4 | 13/24/13 | -    | 5/25/20  | 16/33/1 |
| P value          | 0.322 | 0.130 | 0.285 | 0.262 | 0.486 | 1.00      |       |
| Cyclin D1        | 5/21/24 | 11/34/15 | 17/28/3 | 11/29/10 | 5/25/20 | -       | 16/34/0 |
| P value          | 1.00  | 0.585 | 1.00  | 0.584 | 0.486 | 0.125     |       |
| HER-2            | 13/35/2 | 27/22/1 | 44/6/0 | 32/17/1 | 16/33/1 | 16/34/0 | -     |
| P value          | 1.00  | 1.00  | 1.00  | 1.00  | 1.00  | 0.125     |       |

*P<0.05 was considered as significant. EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; Cox-2: Cyclooxygenase-2; HER-2: Human epidermal receptor-2.*
HER-2 overexpression in the present study was found to be 4%. Reported literature showed overexpression in 13–46.5% of cases \(^{30,34-37}\) and associated with poor prognosis. \(^{36,37}\) However, it was also reported as a rare event with no impact on survival. \(^{33}\) HER-2 positivity was observed in 2/50 cases, and interestingly in both these cases EGFR were also overexpressed. In a previous study, Kaufman \textit{et al}. \(^{32}\) reported that the expression of EGFR was absent in all HER-2 positive cases but due to a small number of patients in that study no conclusive theory was provided. Conversely, in our study both the ERB family of receptors were positive simultaneously, thereby indicating that HER-2 pathway could work in close association with EGFR. This hypothesis needs further evaluation by the large scale prospective studies.

Vascular endothelial growth factor is an important molecule in the angiogenesis pathway and oncogenesis and an essential component in the process of tumor growth and metastatic. VEGF overexpression in the present study was detected in 34% of the cases. Previously, published studies have reported an overexpression ranging from 38% to 75%. \(^{38-41}\) Our results did not confirm the overexpression reported earlier and showed a decreased rate of expression. A possible explanation of variation in frequency could be the geographical and racial distribution and presence or absence of certain unknown carcinogens in this part of the globe that could implicate in the carcinogenesis process involving VEGF. The variation could also be due to the different methods of detection of proteins in the IHC protocol and use of different clones of monoclonal antibodies across the studies. VEGF overexpression in alive cases was significantly high (\(P = 0.044\)) in the present study [Table 2]. Better survival has also been observed in the VEGF positive cases [Figure 2]. Although, our study supports VEGF overexpression corresponds to good survival outcomes, this finding needs to be explored further.

Cyclin D1 is a cell cycle regulatory protein that forms complex with cyclin-dependent kinases that allow cells to go into S phase from G1 phase of cell cycle. Overexpression of cyclin D1 has been observed in 64% of patients in our study, however, previously published studies reported 5.6–41% overexpression and associated with low-grade tumors, papillary tumor morphology and predictors of survival. \(^{28,29,42-44}\) We also observed that overexpression was more in the patients with poorly differentiated tumors (84.6%), and distant metastasis (71.4%), although the result was not statically significant. This could be due to the fact that cyclin D1 influences the cell cycle by exerting its effect on cyclin dependent kinases. In poorly differentiated tumors, cell division is fast which may be due to the accumulation of cyclin D1 that allow transition from one phase of cell cycle to other. However, a contrasting study by Hui \textit{et al}. \(^{43}\) has concluded the defect in the cyclin D1 as an early event in carcinogenesis.

### Table 3: Correlation of Ki-67 labeling index and survival with clinicopathological characteristics

| Variables                      | Ki-67 labeling index (n=50) | Survival (n=46) |
|--------------------------------|-----------------------------|-----------------|
|                                | Mean±SD P value             | Median survival (months) P value |
| Age                            |                             |                 |
| <40                            | 40.0±11.5 0.027             | 23 0.329        |
| >40                            | 27.7±14.6 21                |                 |
| Sex                            |                             |                 |
| Male                           | 28.0±11.6 0.863             | - 0.174         |
| Female                         | 29.7±15.5 16                |                 |
| Tumor stage                    |                             |                 |
| I/II                           | 25.9±15.1 0.261             | 29 0.011        |
| III/IV                         | 31.0±14.4 14                |                 |
| Tubular morphology             |                             |                 |
| No                             | 30.6±14.1 0.337             | 23 0.489        |
| Yes                            | 28.2±15.5 21                |                 |
| Papillary morphology           |                             |                 |
| No                             | 30.0±14.3 0.559             | 19 0.123        |
| Yes                            | 25.7±17.9 -                 |                 |
| Mucinous morphology            |                             |                 |
| No                             | 28.7±15.3 0.156             | 21 0.835        |
| Yes                            | 36.0±5.5 10                 |                 |
| Well differentiated            |                             |                 |
| No                             | 29.8±14.8 0.466             | 19 0.315        |
| Yes                            | 23.3±15.3 21                |                 |
| Moderately differentiated      |                             |                 |
| No                             | 33.8±11.5 0.78              | 13 0.106        |
| Yes                            | 27.4±15.8 22                |                 |
| Poorly differentiated          |                             |                 |
| No                             | 27.0±15.6 0.023             | 22 0.011        |
| Yes                            | 36.2±9.6 10                 |                 |
| Nodal metastasis              |                             |                 |
| No                             | 28.1±14.9 0.452             | 21 0.133        |
| Yes                            | 31.2±14.7 21                |                 |
| Distant metastasis             |                             |                 |
| No                             | 29.7±14.9 0.842             | 21 0.248        |
| Yes                            | 27.9±14.7 10                |                 |
| Status                         |                             |                 |
| Alive                         | 29.1±14.3 0.772             | - -             |
| Dead                           | 28.8±15.0 -                 |                 |

\(P<0.05\) was considered as significant. SD: Standard deviation.
Figure 2: Kaplan–Meier curves for overall survival according to the biomarker overexpression (epidermal growth factor receptor, p53, Bcl2, vascular endothelial growth factor, cyclooxygenase-2, cyclin D1, human epidermal receptor-2) and clinicopathological factors (stage and tumor differentiation)
Our result is in concordance with the previously published reports. The present study provides no significant association of p53 with the clinicopathological factors. However, we noted that in all the well-differentiated tumors, p53 overexpression was negative, and in all the Bcl2 positive cases, p53 was negative. This may be due to the fact that both are apoptotic and anti-apoptotic (reciprocal) proteins. Previous studies have also reported a correlation of p53 expression with clinical stage and survival[28,42,47] in GBC, however, in our study no such association was observed. Furthermore, Bcl2 overexpression in mucinous tumor was statistically correlated (P = 0.045). Mucinous morphology of adenocarcinomas is an advanced and aggressive form of cancer.[46] In our study, the representation of this type of tumor morphology is 5%, which is more than what has been previously reported.[46] As 3 out of 5 mucinous tumors were positive for Bcl2 which are a small number, a clear hypothesis behind this phenomenon cannot be made. We considered strong nuclear staining as positive for p53 expression. It is well-known that p53 gene can be affected by nonsense mutation as well as methylation changes resulting in null expression (no expression of p53 protein). Since such cases were not included as overexpressing p53, therefore this might have resulted in lower rates of p53 alterations in comparison to molecular methods or those studies which have considered completely negative p53 staining (null staining) as positive.

p53 and cox-2 are the molecules closely linked to the tumor formation.[47] Cox-2 is involved in the positive regulation of growth and tumorigenesis, while p53 is involved as a negative regulator of this process. Cox-2 expression in the present study was observed in 66% cases. In the published literature, it was reported as 59.2%, and associated as an early event in carcinogenesis, and also related to the p53 dysregulation.[48] Our study has also shown that cox-2 expression was high in the early stage of tumors (I/II) than the later stages (III/IV) and the result was statistically significant (87.5% vs. 55.9%, P = 0.028). We observed that more than 50% of p53 positive cases were also positive for cox-2 suggesting an association of both the proteins. However, it did not show statistical significance. This observation confirms the previously published report which suggested that cox-2 overexpression may be related to dysfunction of the p53.[49] The co-expression of EGFR and p53 was also observed in the present study, and the result was found to be statistically significant [Table 2]. This observation suggests a close coordination of EGFR receptor pathway and apoptotic pathway which probably work together to exert the overall effect in carcinogenesis.

Ki-67 LI did not provide any correlation with the biomarker expression [Table 1]. However, a previous study by Hui et al.[19] reported that high LI correlated with lymphatic invasion, vascular invasion, and a predictor of early recurrences after surgery. Ki-67 is a proliferative marker. In rapidly dividing cells (in case of carcinoma), it shows higher activity than normally dividing cells. In our study, high LI correlated with the age group <50 years (40.0 ± 11.5, P = 0.027), and poorly differentiated tumors (36.2 ± 9.6, P = 0.023). This indicates that in the poorly differentiated tumor, there is a rapid cell division, and hence, the proliferative activity remains high which was eventually detected as increased in LI. In the younger age, there is increasing and continuous growth and proliferation, thereby showing high LI. Ki-67 LI is also shown as an indicator of progression through the cell cycle.[43]

A study by Artico et al.[49] concluded that Ki-67 expression could be utilized as a prognostic factor for the evaluation of clinicopathological progression. Another study by Hidalgo Grat et al.[50] has not shown any correlation of Ki-67 expression with histological differentiation and survival.

Survival analysis revealed no significant correlation with the biomarker overexpression. These findings are consistent with the already published reports that provided no association of p53, cyclin D1, Bcl2 expression on the survival in GBC.[29] A tendency has been observed that tumors with EGFR or p53 or cyclin D1 or HER-2 overexpression have shown poor survival than other biomarkers of the study. Interestingly, cox-2 positive or VEGF positive or Bcl2 positive tumors showed improved overall survival [Figure 2]. Among clinicopathological characteristics, late stage tumors (stage III/IV), and poorly differentiated tumor have shown poor survival that was also statistically significant on log rank test [Figure 2]. The result is in accordance with the data published by Kim et al.[29]. With such diverse heterogeneous profile of GBC, it will be important to study the molecular mechanism of the disease in order to gain an increased understanding into the insights of GBC.

The study has limitations in terms of its retrospective nature and small sample size, and may have some selection bias.

Overall, the present study is the first of its kind reported from India that involves the role of a panel of biomarkers in GBC and suggests their possible involvement in the development or behavior of this disease. This study reveals that EGFR, cox-2 and cyclin D1 were overexpressed in most of the cases, and HER-2 and Bcl2 overexpression are uncommon in Indian population. p53 was expressed more in poorly differentiated tumors and associated with distant metastasis. Cox-2 overexpression was observed in early stage (I/II) tumors and Bcl2 overexpression in mucinous morphology. EGFR negative, p53 negative, cyclin D1 negative, HER-2 negative, Bcl2 positive, VEGF positive, and cox-2 positive cases have shown a better survival.
Advanced stages of tumor and poorly differentiated tumors are independent predictors of poor survival. Future studies with larger sample size on these lines may provide some important way forward that may be utilized in the diagnosis and treatment of GBC. Further, the panel could be developed for GBC to be used as biomarkers of the disease for risk assessment and development of effective newer targeted agents.

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