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# Prognostic Significance of HER-2 and p53 Expression in Gallbladder Carcinoma in North Indian Patients

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**Key Words**
Gallbladder cancer · HER-2 · p53 · Immunohistochemistry · Survival plots

**Abstract**
***Background/Objective:*** Proto-oncogenes (HER-2) and tumor suppressor genes (p53) are commonly deregulated in gallbladder cancer (GBC). Available literature discloses skewed data from endemic Asian countries, especially north India. This study evaluates the prognostic significance of HER-2 and p53 in GBC patients from two major hospitals. ***Methods:*** Sixty resectable tumor and control specimens were prospectively collected from December 2012 to January 2016. Immunohistochemical staining was done using monoclonal antibodies to semiquantitatively evaluate HER-2 and p53 protein expression. The criterion for HER-2 positivity was set at >30% tumor cells showing complete, membranous staining while p53 positivity was established at <50% tumor cells showing complete nuclear staining. Clinicopathological correlations were drawn with major clinical outcomes. ***Results:*** It was observed that 36.67% (22/60) tumor cases and 5% (3/60) control cases showed strong HER-2 overexpression significantly correlating with sex, T-stage, nodal spread and distant metastasis ($p < 0.05$), while 33.3% (20/60) positivity was observed for p53 in tumor cases and 1.7% (1/60) in control cases. Multivariate analysis showed HER-2 ($p = 0.04$; hazard ratio: 2.36; 95% confidence interval: 1.04–5.33) and p53 ($p = 0.03$; hazard ratio: 5.63; 95% confidence interval: 1.21–26.26) expression to be independent prognostic factors. ***Conclusion:*** Our study thus suggests the plausible role of HER-2 and p53 expression in worse prognosis of GBC in a north Indian population.



## Introduction

Critical data from the Surveillance, Epidemiology, and End Results (SEER) program (1992–2000) show that the incidence of gallbladder cancer (GBC) is assessed at 2.5 per 100,000 persons making it the most common primary biliary tract malignancy, representing 46% of all such malignancies [1]. Major pockets of significantly high oc-

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currences include Central and South America (Chile, Mexico, and Bolivia), Central and Eastern Europe (Hungary, Poland, Czechoslovakia, and Austria) and Japan [2]. The Indian Council of Medical Research (ICMR)-WHO project on the development of a cancer atlas in India has reflected that the occurrence of GBC is predominantly higher in northern India [3]. Recent literature from the reports of the National Cancer Registry Programme of the ICMR suggests that the incidence of GBC corresponds to 3.6 per million in males and 7.4 per million in females in Delhi, India, as compared to 1.13 per million in the US [4, 5]. Women represent almost three fourths of GBC victims and their highest incidence rate globally occurs in Delhi, yet only few common and specific gene studies are available from this high-propensity region [6, 7]. GBC has also been reported to show a variable expression pattern among different ethnic groups.

GBC results via the dysplasia-metaplasia sequence (major pathway) and involves multiple cascades of ERBB and PI3K-AKT signaling among others [8]. The 5-year survival rate for GBC is 32% and for the advanced stage it is only 10% [9]. Current studies have focused on the importance of oncogenes (EGFR, HER-2, K-ras), tumor suppressor genes (p53, FHIT), cell cycle regulators (cyclin D, cyclin E), and micro-RNAs in the development and prognosis of GBC [10]. HER-2 expression evaluation as a biomarker for non-breast cancers has been gaining importance [11]. Among the array of responsible genes, one of the most flagrant genetic aberrations was ascribed to HER-2 in GBC [10]. Yan et al. [12] reported that HER-2 overexpression was detected predominantly in malignancies of epithelial origin. Furthermore, the availability of FDA-approved agents targeting HER-2-positive malignancies, such as trastuzumab and pertuzumab (humanized monoclonal antibodies) and lapatinib and afatinib (dual EGFR/HER2 inhibitors), makes it a marker of choice for testing in GBC.

The PI3K/Akt/mTOR pathway has been closely linked to the pathogenesis of numerous carcinogenic conditions. Deregulation of PI3K signaling as a consequence of PIK3CA mutations or PTEN loss has been described in 5–12 and 50% of human GBC, respectively [13]. Genotoxic as well as nongenotoxic inflictions to the cells give rise to the activation of the p53 gene, which acts as a gatekeeper gene to watch cell proliferation against cancer. Mutated p53 proteins have a longer half-life as compared to their wild-type counterparts and can be easily assessed by standard immunohistochemistry methods.

However, concrete results have not been obtained due to a limited number of resectable GBC cases presented at the hospital. We, therefore, attempt to evaluate the immunoexpression of HER-2 and p53 receptors in 60 resectable prospective GBC cases across two major hospitals based in Delhi, India, and determine their prognostic value in the selected GBC cohort.

**Table 1.** Population characteristics

| Factor | Category | Population fraction (n = 60) (%) |
|---|---|---|
| Age | <50 years old | 15 (31.25) |
| | >50 years old | 33 (68.75) |
| Gender | Male | 15 (25) |
| | Female | 45 (75) |
| Presence of stones | Negative | 20 (33.33) |
| | Positive | 40 (66.67) |
| Macroscopic type | Mass forming | 24 (40) |
| | Non-mass forming | 36 (60) |
| Lymph node metastasis (N) | Negative | 43 (71.67) |
| | Positive | 17 (28.33) |
| Pathological grade (T) | 1+2 | 27 (45) |
| | 3+4 | 33 (55) |
| Distant metastasis (M) | Absent | 46 (76.67) |
| | Present | 14 (23.33) |
| Histological classification | Well-differentiated | 17 (28.33) |
| | Moderately differentiated | 25 (41.67) |
| | Poorly differentiated | 18 (30) |
| Lymphovascular invasion | Negative | 34 (56.67) |
| | Positive | 26 (43.33) |
| Perineural invasion | Negative | 36 (60) |
| | Positive | 24 (40) |

## Methods

*Tissue Specimens*

Sixty freshly resected GBC cases were collected from the G.B. Pant hospital (associated with Maulana Azad Medical College), and HAH centenary hospital from Delhi, India, after obtaining ethical clearance from both the hospitals and informed consent from patients. As control, noncancerous adjacent tissues from each patient were collected routinely from the surgery department of the participating hospitals. Also, a structured questionnaire was prepared to obtain information on demographic, clinical, and epidemiological factors. None of the patients had received any radio- or chemotherapy before surgery. Histological type, differentiation grade, and TNM (tumor node metastasis) staging of all specimens was obtained from H&E-stained slides

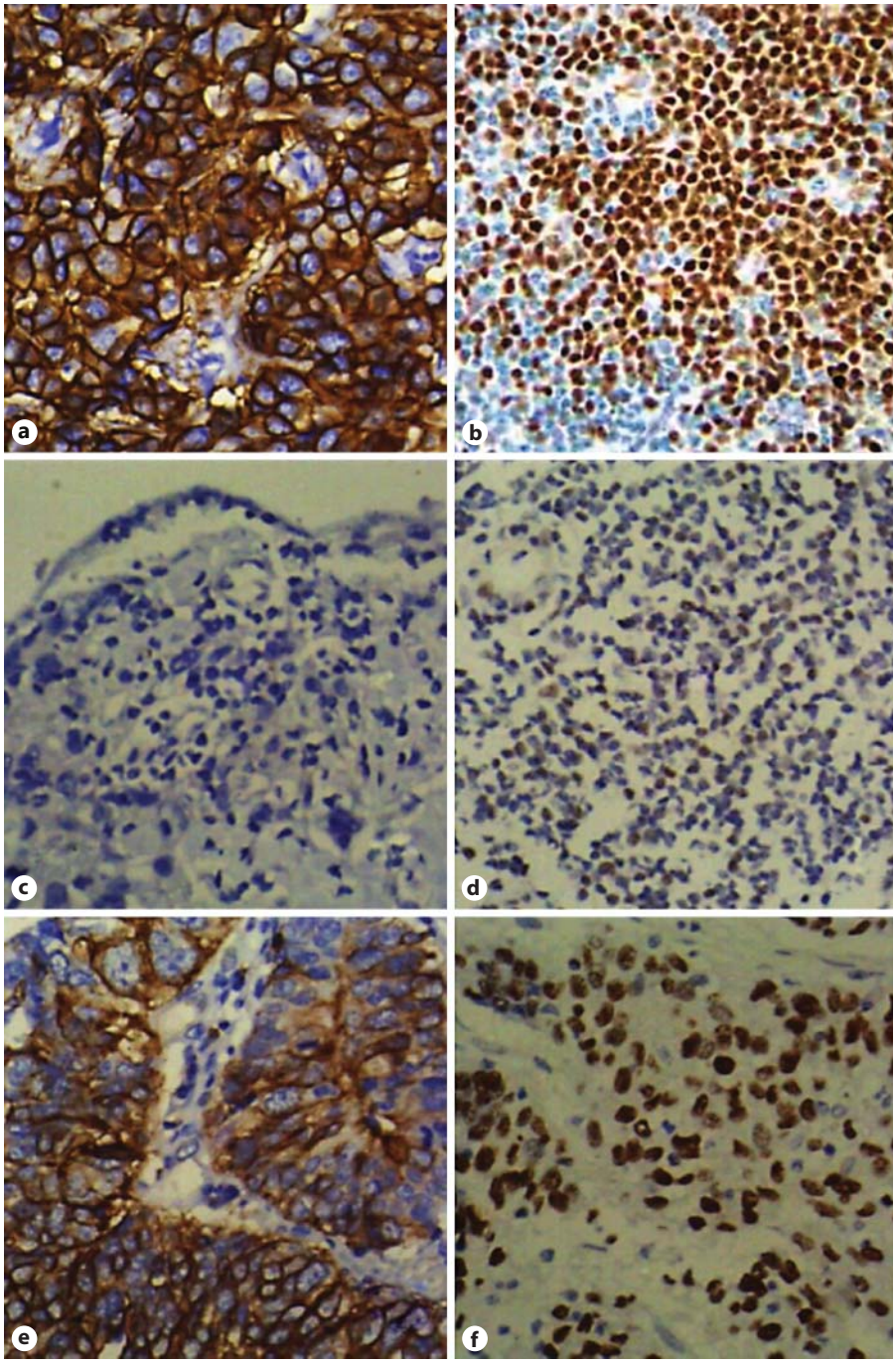


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**Fig. 1.** Scoring scheme for HER-2 and p53 staining. Positive control (×100) (**a**, **b**), negative staining at ×100 (0) (**c**, **d**) and strong positive, complete membranous staining at ×100 (+2) (**e**, **f**).

and hospital records. Patients' follow-up statuses were obtained either at a follow-up visit or through contact with the patients or their relatives. Clinicopathological features of all patients are summarized in table 1.

*Immunohistochemistry*

Formalin-fixed (10%), paraffin-embedded GBC and control specimens were sectioned (3–5 μm thick) and placed on polylysine slides. The acquired sections were initially dewaxed using xylene and rehydrated in alcohol followed by immersion in 1.5% $H_2O_2$ in methanol for about 10 min. Washing was done with tap water to eliminate excess peroxide and the slide with normal rabbit sera was incubated for 10 min to block nonspecific binding. Residual serum was removed and the sections were rapidly treated with ready-to-use primary antibodies and incubated for 30 min in a humid chamber. Monoclonal antibodies c-erbB-2 (anti-HER-2) and DO-7 (anti-p53) (Dako® Corporation, Carpinteria, Calif., USA) were used as the primary antibody. Prediluted (1:1,600) biotinylated second-

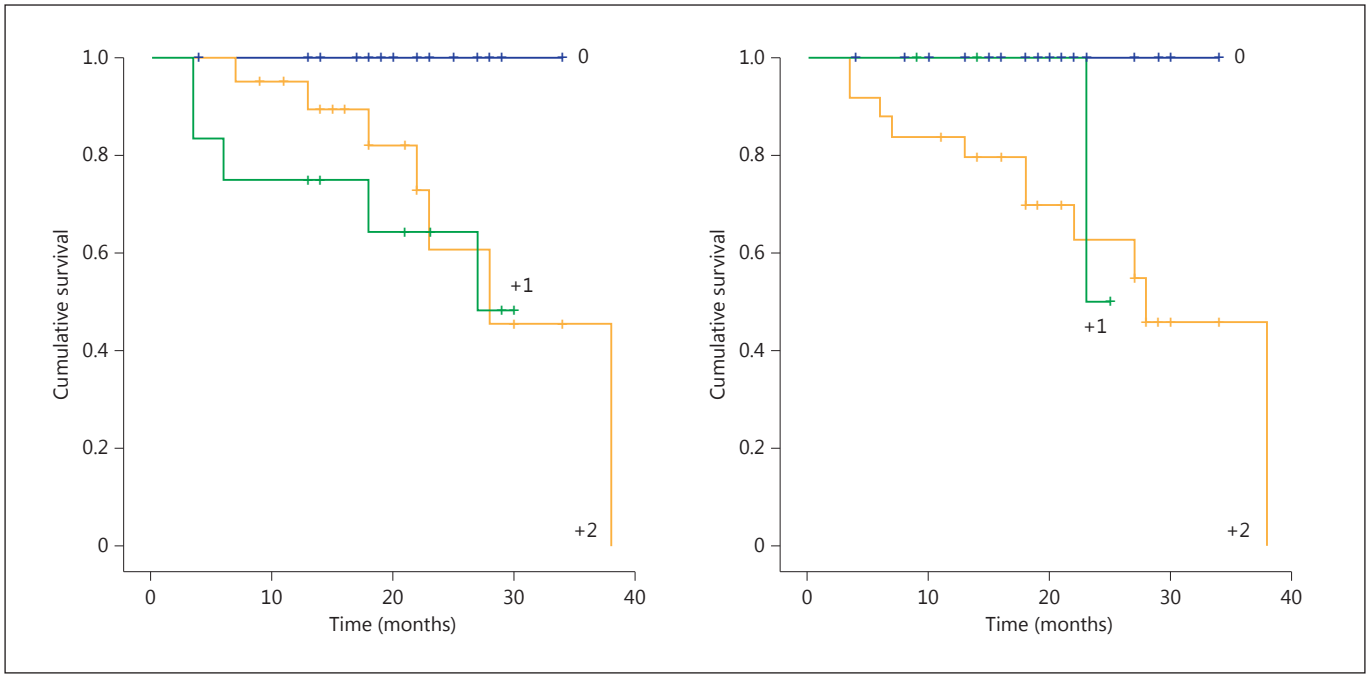


**Fig. 2.** Kaplan-Meier survival plots for HER-2 and p53 expression (control and tumor samples at different scores).

ary antibodies (horseradish peroxidase, Vector Laboratories Inc., Burlingame, Calif., USA) were applied to the sections for an hour after washing the slide with Tris buffer (pH 7.6). The slides were repeatedly washed with Tris buffer saline for 10 min and finally the color was developed by the application of diaminobenzidine as the chromogen. Slides were then washed with tap water to arrest the biochemical processes followed by counterstaining with Harri's hematoxylin for a couple of minutes. The specimens were then washed, dried in alcohol and mounted with DPX.

*Scoring*

Scoring was based on the Herceptest™ (Dako) criteria; semi-quantitative analysis of the stain intensity was carried out [14]. The number of HER-2-stained cells in 10 representative microscopic fields were counted and the percentage of positive cells was adjudged as negative (0) or barely perceptible staining in <10% tumor cells (0), weak staining in <30% tumor cells (+1), strong, complete membranous staining in >30% cells (+2) while p53 positivity was established when more than 50% tumor cells exhibited complete nuclear staining (+3) [15].

*Statistics*

Statistical analysis was performed with two-sided $\chi^2$ test using SPSS software 23.0. Fisher's exact test was used for the analysis of contingency tables and evaluation of variance for the averages. Cumulative survival rates and survival curves were calculated by Kaplan-Meier actuarial survival curves and log-rank tests. The Cox-proportional hazards model was used to identify the hazard ratio (HR) and 95% confidence interval (CI) of the clinical outcomes along with significant covariates for negative and positive patient groups. Statistical significance was set at $p < 0.05$.

## Results

The average age of the patient cohort was 52.34 ± 10.84 years, and 75% of them were females. 66.7% of cases reportedly had gallstones of variable sizes and the median tumor size was 30.68 ± 22.55 mm. All the cases were invariably histologically proven adenocarcinoma. Nearly 45% of the cases belonged to T-stages I and II, 28.33% of the patients showed lymph node positivity (N) and 23.33% had metastatic behavior. About 36.67% (22/60) of the patients showed complete membranous staining for HER-2 as against only 5% (3/60) of control cases ($p < 0.05$) and 33.3% (20/60) of the tumor cases showed positivity for p53 while 1.7% (1/60) of the control cases showed complete nuclear staining (fig. 1). Contingency table analysis by $\chi^2$ tests revealed that HER-2 positivity showed significance with sex ($p = 0.02$), T-stage ($p = 0.03$), nodal metastasis N ($p < 0.001$) and distant metastasis M ($p < 0.001$). On the other hand, p53-positive cases did not show any significance with clinicopathological factors. Univariate analysis using Kaplan-Meier survival plots revealed that nodal metastasis ($p < 0.001$), HER-2 expression ($p < 0.05$) and p53 expression ($p < 0.05$) showed statistical significance (fig. 2). Application of the Cox-proportional hazards model (multivariate analysis) indicated HER-2 ($p = 0.04$; HR: 2.36; 95% CI: 1.04–5.33) and p53

**Table 2.** Univariate and multivariate analysis of survival (months) with GBC

| Parameter | Univariate | | Multivariate | |
|---|---|---|---|---|
| | 95% CI of mean | p value[a] | HR of death (95% CI) | p value[b] |
| Gender | | 0.38 | 2.50 (0.30–20.65) | 0.35 |
| Male | 27.26–39.74 | | | |
| Female | 23.64–31.42 | | | |
| Gallbladder stones | | 0.72 | 1.23 (0.30–5.54) | 0.72 |
| Present | 24.11–35.84 | | | |
| Absent | 24.39–33.69 | | | |
| Histology | | 0.88 | 1.13 (0.45–2.85) | 0.79 |
| G1 | 25.76–38.76 | | | |
| G2 | 22.25–33.99 | | | |
| G3 | 23.22–31.35 | | | |
| Lymph node (N) | | 0.05 | 3.90 (0.86–17.68) | 0.08 |
| Present | 18.33–30.01 | | | |
| Absent | 30.12–38.24 | | | |
| Pathological grade (T) | | 0.37 | 0.45 (0.07–2.70) | 0.38 |
| I+II | 19.11–27.10 | | | |
| III+IV | 26.46–35.23 | | | |
| Distant metastasis (M) | | 0.99 | 0.99 (0.12–8.31) | 0.99 |
| Present | 29.00–29.00 | | | |
| Absent | 26.23–35.88 | | | |
| Lymphovascular invasion | | 0.87 | 0.87 (0.16–4.83) | 0.87 |
| Present | 27.52–29.48 | | | |
| Absent | 26.48–37.12 | | | |
| Perineural invasion | | 0.51 | 1.71 (0.34–8.59) | 0.51 |
| Present | 24.15–30.23 | | | |
| Absent | 25.74–34.13 | | | |
| HER-2 expression | | 0.05 | 2.83 (1.16–6.88) | 0.04 |
| 0 | – | | | |
| +1 | | | | |
| +2 | | | | |
| p53 expression | | 0.02 | 51.82 (0.13–20,340) | 0.03 |
| Present | – | | | |
| Absent | | | | |

[a] p value by log-rank test. [b] p value by the Cox-proportional hazards test.

(p = 0.03; HR: 5.63; 95% CI: 1.21–26.26) expression to be significant prognostic factors in the selected GBC cohort (table 2).

## Discussion

GBC is a neoplasm pertinently prevalent in select parts of the world. The majority of investigations that have been reported vary greatly due to geographical and racial disparities. North India presents itself as a major hub for this deadly cancer with a varied gene and protein expression profile. The pathogenesis of GBC is a much talked about phenomenon and often reports the involvement of major proto-oncogenes such as EGFR, HER-2 and tumor suppressor genes such as p53 [16]. In this study, we therefore attempt to identify the immunoexpression of HER-2 and p53 receptor proteins in 60 GBC patients. The primary reasons for skewed results obtained from the same as well as different regions can be attributed to the source of antibodies used for immunohistochemistry, race of the patient cohort, and criteria used to adjudge positivity in the stained samples [17].

A number of recent studies have identified ERBB signaling to be the most frequently mutated pathway [18] in GBC (36.8%) giving rise to their oncogenic potential and underlines the significance of HER-2 in GBC development and progression. However, in a rather unusual

study, Toledo at al. [19] reported the overexpression of HER-2 as an early event in intestinal metaplasia (92%), carcinoma in situ (90%) and invasive GBC (33%). In our preliminary investigation, the HER-2 expression profile showed relevance in higher-stage cases and significance with T-stage and nodal spread (N) of the disease, which is often seen in advanced GBC cases. In a rather recent study conducted by Yoshida et al. [20], they found a significant patient population that can derive benefit from anti-HER-2 therapy by designing planned clinical trials based on preliminary immunohistochemical reports.

Several human cancers have reportedly shown the involvement of the PI3K/Akt/mTOR pathway in the pathogenesis with remarkable effects on cell cycle progression, cell proliferation, and angiogenesis. Deregulation of PI3K signaling as a consequence of PIK3CA mutations or PTEN loss has been described in 5–12 and 50% of human GBC, respectively [13]. Tian et al. [21] investigated the involvement of the p53 gene in regulating tumor angiogenesis and correlated it with tumor progression and increased vascularity. However, Chaube et al. [22] in their study regarded it to be involved in early phases of GBC while the majority of other studies considered it to be otherwise. Shu et al. [23] conducted an immunohistochemical study of benign and malignant lesions of GBC and also suggested the prognostic role of p53. We found that p53 expression was observed in almost one third of the selected patient cohort and presented itself along with HER-2 as an independent prognostic factor indicating worse prognosis. In the past, the treatment for all sorts of biliary tract cancers used to be identical. However, improvements in next-generation sequencing and other molecular techniques, and differentiation between these tumor types have confirmed that each tumor type (GBC, intrahepatic cholangiocarcinoma, and extrahepatic cholangiocarcinoma) has a unique molecular signature. It is therefore important for identifying drugs targeting specific pathways that can be utilized in biomarker-driven clinical trial designs. Therefore, preliminary studies from endemic regions for GBC such as India are critical to define prospective patient subsets that may derive benefit from targeted therapy.

Consequently, it can be summed up that there is an imperative necessity to search for archetype markers of GBC, which will not only diagnose and prognosticate the disease but also help in choosing the apposite modality of therapy, which may give us an opportunity to make headway in our basic understanding of GBC biology and management paradigms. Furthermore, an accurate molecular characterization of tumors is necessary for the enrolment of patients in clinical trials in order to define which patient groups are likely to gain the most from targeted therapy. Multiple cellular pathways influence the growth and metastatic potential of tumors creating heterogeneity, termination, and the potential for tumors to overcome the signaling pathway blockade, resulting in primary or acquired resistance. Combining therapies that inhibit different intracellular signaling pathways have the potential to be more effective than inhibition of a single pathway and overcoming tumor resistance. A new promise in gallbladder treatment is the inhibition of the master heat shock protein 90, a protein that in the last decade has emerged as an exciting target for cancer therapy, which is a master regulator of the stability and activity of multiple oncoproteins such as p53, HER-2, Akt, Bcr-Abl, c-Kit, EGFR and mutant BRAF [24]. Thus, our study assesses the expression of two major genes involved in the pathogenesis of GBC, correlates it with clinicopathological parameters and establishes it as an independent prognostic factor in GBC.


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## Disclosure Statement

The authors declare no conflict of interest.

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