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Original Article

Pathology Section

Role of Epidermal Growth Factor Receptor in Gallbladder Carcinoma and its Association with Various Clinicopathological Parameters: A Cross-sectional Study

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ABSTRACT

Introduction: Carcinoma of gallbladder is an aggressive disease with poor outcome. The Epidermal Growth Factor Receptor (EGFR) is a transmembrane receptor that regulate growth, proliferation and differentiation in cells. Increased EGFR receptor expression has been studied in various cancers like lung, colorectal, breast and pancreatic tumours and anti-EGFR antibody has been used successfully for therapeutic and diagnostic purposes.

Aim: To evaluate EGFR expression in gallbladder carcinoma and its association with clinicopathological factors to reveal its relation to prognosis.

Materials and Methods: This cross-sectional retrospective observational study in which 64 samples were collected of resected specimen of Gallbladder Carcinoma (GBC), from the Department of Pathology, King George's Medical University, Lucknow, Uttar Pradesh, India, between March 2017 to September 2018. Haematoxylin and Eosin (H&E) stained sections were evaluated for tumour type and histopathological grading and TNM staging was done. Immunohistochemistry (IHC) was performed and analysed prospectively using ready to use anti-EGFR as per manufacturer's protocols. Association between EGFR expression and clinicopathological factors were statistically analysed using Statistical Package for the Social Sciences (SPSS) software version 21.0.

Results: The EGFR expression was positive in 52 (81.25%) cases of GBC which showed a highly significant association between tumour grade and stage. About 60% of poorly differentiated GBC displayed strong (3+) staining intensity as compared to 30% of moderately differentiated (3+) and 10% of well differentiated tumours (3+). It suggests that with decreasing differentiation of tumour EGFR staining intensity increases ($p<0.001$). Positivity rate of EGFR expression were also increased with increase of tumour TNM stage (stage I to stage IV). Strong EGFR expression was associated with decreased overall survival with significant p-value ($p=0.031$, log rank test).

Conclusion: The EGFR expression is inversely related with tumour differentiation, and overall survival. EGFR expression increases with high TNM staging. So, it can be used as prognostic marker for gallbladder carcinoma and opening a hope towards the new therapeutic options.

Keywords: Adenocarcinoma, Gallbladder cancer, Immunohistochemistry, Prognostic marker

INTRODUCTION

Gallbladder Carcinoma (GBC) is the most common malignancy of the biliary tract, accounting for 80-95% of biliary tract cancers worldwide. It is two times more common in females than males [1,2]. Incidence broadly vary with the variation of geography, ethnicity and cultural differences. It suggests the key role of genetic and environmental factors in the development and progression of GBC. Indo-Gangetic belt particularly northern India and south Karachi in Pakistan are one of the highest affected regions in the Asia [2,3]. Most cases of GBC are diagnosed at advanced stage of disease when patients present with obvious sign and symptoms. They have high recurrence rate and poor prognosis [1,4].

Hence, key to improve the outcome of GBC, early diagnosis and intervention are needed. So, it is very important to recognise molecules which are involved in tumour progression and proliferation of GBC. EGFR is a transmembrane receptor belonging to the ErbB family of receptor tyrosine kinases. On activation, receptor dimerisation occurs, which signals within the cell by causing receptor autophosphorylation. It triggers in a chain of intracellular pathways that possibly result in cancer-cell proliferation, blocking apoptosis, activating invasion and metastasis and capillary formation [5-7]. Increased EGFR expression has been reported in various cancers such as colon, squamous cell of the head and neck, non small cell lung and breast cancers in different region of the world and anti-EGFR antibody has been used as target therapy [1,7-10]. Limited studies have been done so far in the Indian subcontinent on expression of EGFR in gallbladder malignancies.

The present study was conducted to observe the EGFR expression in GBC and to find its association with histopathological grades and various clinicopathological factors to reveal its relation to prognosis.

MATERIALS AND METHODS

This cross-sectional retrospective observational study was conducted at the Department of Pathology in collaboration with Department of Surgical Gastroenterology King George's Medical University, Lucknow, Uttar Pradesh, India. The duration of study and analysis was one year and six months (March 2017 to September 2018). Study was approved by the Institutional Ethical Committee with ethical number 803/Ethic/R.cell-18.

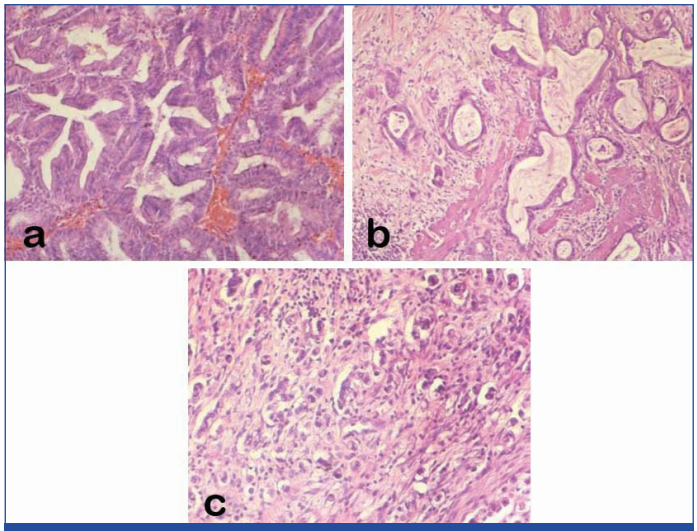
Inclusion criteria: The sample included patients having primary GBC.

Exclusion criteria: Samples excluded who had metastatic carcinoma, post chemotherapy and post radiotherapy of GBC.

Study Procedure

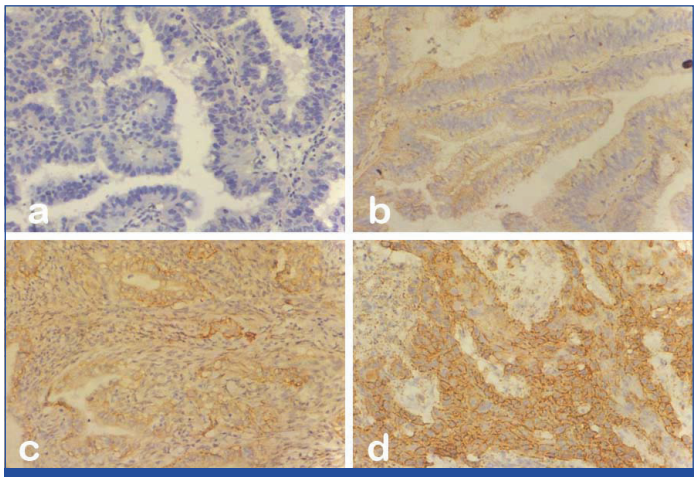
Details of each patient related to their demographic profile, investigations, tumour profile including lymph node status,

histopathology types were documented. Follow-up information were recorded from the medical records as well as by telephonic calls of all patients till end of the study. Out of 64 patients, 14 patients lost the follow-up and 50 patients were available for survival analysis. After tissue processing, 3-4 µm thick sections were cut. Histopathological evaluation was performed on Haematoxylin and Eosin (H&E) stained sections. GBC cases were further categorised on the basis of histological types, grading and TNM tumour staging of American Joint Committee on Cancer (AJCC) [11,12]. A GBC tumour was labelled as well-differentiated if gland formation was predominant and cytological atypia was not pronounced and as poorly differentiated when it was arranged in sheets with only occasional glandular component. The cases having features in between of the above two was designated as moderately differentiated adenocarcinoma [Table/Fig-1].



[Table/Fig-1]: Histological picture of gallbladder carcinoma; a) Well differentiated adenocarcinoma (H&E, 100X); b) Moderately differentiated adenocarcinomax (H&E, 100X); c) Poorly differentiated adenocarcinoma (H&E, 100X).

The IHC was performed using ready to use anti- EGFR (manufactured by Biogenix) as per manufacturer's protocols. Scoring of EGFR expression was done by guidelines based on study by Kaufman M et al., [6]. The immunostaining was considered positive only when membranous or membranous with cytoplasmic in location. According to intensity a score of 1+, 2+, and 3+ were assigned when staining intensity was weak, moderate and strong, respectively whereas score 0 was assigned for no staining [Table/Fig-2].



[Table/Fig-2]: a) No absence of immunostaining for EGFR (0) 200X; b) Positive immunostaining for EGFR weak (1+) 100X; c) Positive immunostaining for EGFR moderate (2+) 100X; d) Positive immunostaining for EGFR strong (3+) 200X.

STATISTICAL ANALYSIS

Data analysis was done using SPSS version 21.0. Data was represented in number, percentage mean and Standard Deviation (SD). Quantitative variables were compared using Unpaired t-test/ Mann-Whitney test. Qualitative variables compared using Chi-square test/Fisher's exact test as suitable. Disease specific overall survival period was analysed and compared using the Kaplan-Meier method and the log-rank test. A 'p' value of <0.05 was considered statistically significant.

RESULTS

In this study, age range of the patients was 26-70 years with mean age of 50.12±11.55 years. Majority of the patients were females 50 (78.1%) cases with a male to female ratio of 1:3.6. The distribution of cases into well moderate and poorly differentiated adenocarcinoma is shown in [Table/Fig-3].

S. No.	Histological grade	Total n cases	Percentage (grade wise) (%)
1.	Well Differentiated (WD)	23	35.9
2.	Moderately Differentiated (MD)	25	39.1
3.	Poorly Differentiated (PD)	16	25
Total		64	100

[Table/Fig-3]: Distribution of sample according to histological grade.

In this study EGFR expression was positive in 52 (81.25%) cases of GBC. On the basis of staining intensity positive cases were further categorised into week (1+), moderate (2+) and strong (3+). Total 22 (42.3%) cases showed moderate (2+) intensity, 20 (38.5%) strong (3+) and 10 (19.2%) showed weak (1+) EGFR staining intensity [Table/Fig-2].

The positivity rate of expression of EGFR was compared to the degree of differentiation of tumour and it was observed that 60% of poorly differentiated GBC displayed strong (3+) staining intensity as compared to 30% of moderately differentiated and 10% of well differentiated tumours [Table/Fig-4]. These findings suggest that with decreasing differentiation of tumour EGFR immunostaining intensity increases (p<0.001).

Histological grades	Total	EGFR immunostaining			
		Negative	Positive		
		0	1+ (weak)	2+ (Moderate)	3+ (Strong)
Well differentiated (WD)	23	9 (75%)	6 (60%)	6 (27.3%)	2 (10%)
Moderately differentiated (MD)	25	3 (25%)	3 (30%)	13 (59.1%)	6 (30%)
Poorly differentiated (PD)	16	0	1 (10%)	3 (13.6%)	12 (60%)
Total	64	12	10	22	20

[Table/Fig-4]: Association of EGFR expression with different histopathological grades of GBC.
χ^2=29.44; p=0.001

The EGFR expression was also association to various histological types of gallbladder carcinoma, like intestinal, mucinous, papillary, signet ring cell, clear cell adenocarcinoma and was found to give significant association among various histological types of GBC [Table/Fig-5].

Total 60 cases were evaluated (rest four were biopsy case) for staging according to TNM classification of tumours. In stage I: maximum 50% cases were negative for EGFR, 20% cases showed weak (1+) EGFR expression I while in stage II: 33.33% negative for EGFR and 31.82% showed strong (2+) expression and in stage III: 16.67% negative and 45.45% showed moderate (2+) staining and in stage IV although cases are very less (due to high mortality) they showed 100% positivity rate [Table/Fig-6]. This data found to be statistically significant (p=0.035).

There was no significant association between EGFR and other clinicopathological factors like age, sex, gall stones, lymph node metastasis and surrounding tissue invasion [Table/Fig-7].

Type	Total	EGFR expression			
		Negative n (%)	1+ (Weak) n (%)	2+ (Moderate) n (%)	3+ (Strong) n (%)
Not otherwise specified	41	6 (14.6)	4 (9.8)	17 (41.5)	14 (34.1)
Papillary	8	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)
Mucinous	7	1 (14.3)	2 (28.6)	3 (42.8)	1 (14.3)
Signet cell	1	0	0	0	1 (100)
Intestinal	3	2 (66.7)	0	1 (33.3)	0
Clear cell	1	0	1 (100)	0	0
Adenos-quamous	3	0	0	0	3 (100)
Total	64	12	10	22	20

[Table/Fig-5]: EGFR positivity rate in different histological types of gallbladder. χ^2=28.84; p=0.05

Stage	Total cases (60)	EGFR immunostaining				'p'-value
		Negative cases (12)	Positive cases (48)			
			1+ (weak) (10 cases)	2+ (Moderate) (22 cases)	3+ (Strong) (16 cases)	
I	12	6 (50%)	2 (20%)	4 (18.18%)	-	0.035
II	26	4 (33.33%)	5 (50%)	7 (31.82%)	10 (62.50%)	
III	19	2 (16.67%)	2 (50%)	10 (45.45%)	5 (31.25%)	
IV	3	-	1 (10%)	1 (4.55%)	1 (6.25%)	

[Table/Fig-6]: EGFR expression in various TNM stages of gallbladder carcinoma. χ^2=18.009; p=0.035

Clinico-pathological factors	EGFR interpretation		χ^2 value	'p'-value
	Negative n (%)	Positive n (%)		
Sex				
Male (n=14)	4 (33.33)	10 (19.23)	3.6	0.308
Female (n=50)	8 (66.67)	42 (80.76)		
Age criteria				
≤50 (n=37)	9 (75.0)	28 (53.85)	2.59	0.459
>50 (n=27)	3 (25.0)	24 (46.15)		
Gall stones				
Absent	4 (33.33)	28 (53.85)	3.556	0.314
Present	8 (66.67)	24 (46.15)		
Lymph node metastasis				
No	9 (75.0)	23 (47.92)	8.256	0.264
Yes	3 (25.0)	25 (52.02)		
Surrounding tissue invasion				
No	11 (91.7)	36 (69.23)	3.213	0.360
Yes	1 (8.3)	16 (30.77)		

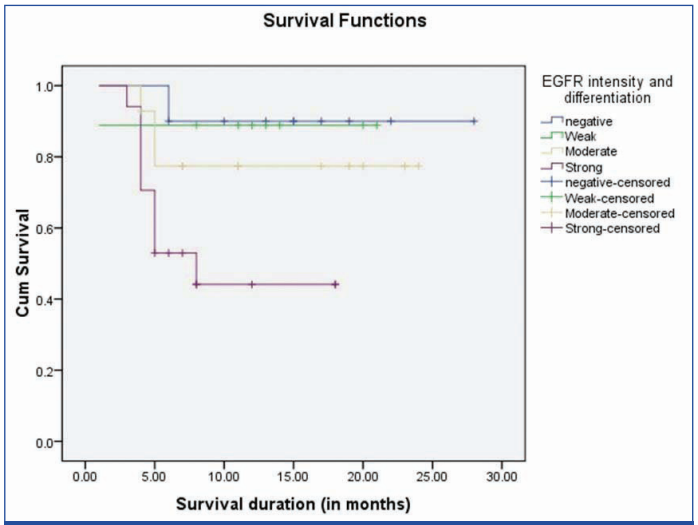
[Table/Fig-7]: Association of EGFR expression with various clinicopathological factors.

EGFR expression in terms of mean survival of the patients: The mean survival time was 9.36±7.82 months. Total 50 cases were followed till the end of study. Among them, 26 patients were survived more than nine months had EGFR positive rate of 69.23% whereas 24 patients were died within nine months had with EGFR positive rate of 91.67% and it was found statistically significant (p=0.048) [Table/Fig-8].

Survival	EGFR interpretation			χ^2 value	'p'-value
	Total (50)	Positive (40)	Negative (10)		
<9 months	24	22 (91.67%)	2 (8.33%)	3.926	0.048
≥9 months	26	18 (69.23%)	8 (30.77%)		

[Table/Fig-8]: Association of EGFR expression with survival in GBC cases.

Survival graph: Kaplan-Meier plots for overall survival in 50 patients with GBC in relation to EGFR expression showed that strong EGFR expression was associated with decreased overall survival with significant p-value (p=0.031, log rank test) [Table/Fig-9].



[Table/Fig-9]: Kaplan-Meier survival graph.

DISCUSSION

Carcinoma of gallbladder is a very aggressive disease with early spread to liver and surrounding area [1,4]. Early diagnosis and complete surgical resection are the only hope for long-term disease-free survival. However, only 0-15% GBC cases present in early-stage, which were considered for surgery [1,7,8]. Patients with unresectable or metastatic disease have a poor prognosis. it is essential to develop other therapeutic options for these patients [8,9]. EGFR is a signalling pathway that regulate growth, proliferation and differentiation in cells [5,7,9]. It had been studied in head neck cancer, colon, breast and lung cancer and EGFR expression, represents aggressive and rapidly growing property in a tumour [9,10].

In present study, 35.1% cases of GBC were well-differentiated adenocarcinoma, 39.1% were moderately differentiated and 25% were poorly differentiated. These results were in concordance with Brandt-Rauf et al., who also found that the most common neoplasms were moderate to well-differentiated (40–50%), while poorly differentiated was 30% [10].

Among overall positivity rate of EGFR, 19.2% cases showed weak (1+), 42.3% moderate (2+) and 38.5% cases displayed strong (3+) EGFR immunostaining. This was similar to study of Kaufman M et al., and they also found a predominance of EGFR overexpression (93.75%) in GBC cases in which, 18.75% cases were 1+, 56.3% were 2+ and 18.75% were 3+ on immunohistochemistry [6].

On observing the degree of differentiation and intensity of EGFR, strong (3+) EGFR immunostaining intensity was found in 10% well-differentiated, 30% moderately differentiated and 60% poorly-differentiated tumours. This finding demonstrated that with decreasing differentiation of the tumour, EGFR immunostaining intensity was increased. It suggests an inverse relationship between tumour differentiation and EGFR expression. Since, poorly differentiated tumours behave more aggressively, the intensity of EGFR expression may associate with aggressiveness of disease. This finding was similar to North American study by Kaufman M et al., and Indian studies by Kumar N et al., Hadi R et al., and Doval DC et al., [6,13-15]. They also reported an inverse relationship between tumour differentiation

and EGFR expression in adenocarcinoma gallbladder. Kumar N et al., found that most of the specimen showing weak EGFR immunostaining intensity (1+) were well-differentiated tumour 7/10 (70%) and strong EGFR immunostaining intensity (3+) were poorly differentiated cases of adenocarcinoma (6/8, 75%) [13]. Variants of gallbladder adenocarcinoma were also studied and classified them according to the World Health Organisation (WHO) classification [11]. It was found that most common histological type was adenocarcinoma NOS (41 cases) followed by papillary (eight cases) and mucinous adenocarcinoma (seven cases). There was no significant association was found between EGFR expression and histological types which was similar to finding of Hadi R et al., [14]. EGFR expression were also associated with TNM tumour staging and observed that from stage I to IV, the positivity rate of EGFR expression increases from 50-100%. The cases showed strong (3+) immunostaining increased from 0-31% with increased tumour stage, which denotes poor prognosis. It was similar to findings of Doval DC et al., who reported that EGFR is largely overexpressed in advanced tumour stages and poorly differentiated tumours which are predictors of poor survival [15]. In another study by Martins SJ et al., and Viswanath S et al., reported that advanced stage GBC was associated with overexpression of EGFR [16,17]. Das C et al., and Zhou YM et al., observed that EGFR is involved in gallbladder carcinogenesis and is related to high proliferative activity and aggressive nature of the tumour [18,19].

On observing the EGFR expression with various clinical and pathological factors, no significant association was observed between EGFR expression and prognostic factors like gallstone, lymph node metastasis and surrounding tissue invasion. However, Martins SJ et al., reported a significant correlation between EGFR overexpression and lymph node metastasis [16]. An inverse relationship between EGFR expression and overall survival of GBC patients was found. Short survived group (<9 month) showed 22/24 (91.67%) expression for EGFR as compared to 18/26 (69.23%) in long survived group (>9 months). The cases showing strong (3+) EGFR staining were survived lesser as compared to 1+ or negative cases. This was similar to the finding of Martins SJ et al., who found that tumour immunoreactivity for EGFR in nearly half of the cases which was independently associated with poor survival in GBC patients [16]. Pais-Costa SR et al., Pignochino Y et al., and Shafizadeh N et al., analysed multiple markers in GBC and summarised that worse prognosis was related to increased immunoexpression of the protein EGFR in the tumour tissue [20-22]. Lee CS and Pirdas A studied EGFR receptor immunoreactivity in gallbladder and extrahepatic biliary tract tumour and found that EGFR overexpression occurs late in the sequential development of ball bladder and biliary tract cancers [23]. In present study, EGFR positivity rate was 81.25% in GBC. This finding was similar to previous studies reported in the literature [Table/Fig-10] [6,13-15,18,19,21-23].

Studies	Number of GBC cases	EGFR expression (%)
Das C et al., [18] (2021)	30	93.33
Kumar N et al., [13] (2016)	50	88
Hadi R et al., [14] (2016)	18	77.78
Doval DC et al., [15] (2014)	50	74
Pignochino Y et al., [21] (2010)	13	38.5
Safijadeh N et al., [22] (2010)	50	80.00
Kaufman M et al., [6] (2008)	16	93.75
Zhou YM et al., [19] (2003)	41	71
Lee CS and Pirdas A [23] (1995)	13	100
Present study (2022)	64	81.25

[Table/Fig-10]: EGFR expression in GBC of previous studies [6,13-15,18,19, 21-23].

Limitation(s)

This study has small sample size with short duration. Further studies should be conducted on large sample size in order to validate it as prognostic and therapeutic molecule.

CONCLUSION(S)

Gallbladder carcinoma is an aggressive malignancy with overall poor survival. EGFR expression is inversely related with tumour differentiation. It is associated with aggressive disease and decrease overall survival. It may serve as a prognostic marker and also as target for molecular therapy in gallbladder carcinomas.

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