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Original Study

EGFR and HER–2/neu Expression in Gallbladder Carcinoma: An Institutional Experience

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Abstract

Background

Gallbladder cancer (GBC) is the most common malignancy of the biliary tract in India. Despite the recent advancement in the understanding of the cancer biology, the disease still remains a therapeutic challenge with poor prognosis and low survival. Surgery is the primary modality of treatment and rest of the modalities are basically adjuvant in nature. This study was performed to evaluate the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor–2 (HER–2/neu) expression in GBC.

Methods

The present study was prospective and done in tertiary super–specialty institute of northern India. This was a pilot study, and at the time of completion, 29 samples were found suitable which were later submitted for EGFR and HER–2/neu evaluation. The sample includes both cases of GBC (n=18) and cholecystitis (n=11) as control. (Table 1) After performing necessary processing, slides were incubated with primary antibody (EGFR) ready to use (RTU) BioGenex, India and HER–2/neu dilution 1:600, Dakopatts (Denmark). Then slides were incubated with secondary antibody (Real Envision Detection Kit, Dakopatts, Denmark). Finally in mounted slides, cell membrane staining was used to assess positivity for EGFR and HER–2/neu.

Results

EGFR was positive in 21/29 (72.41%) overall, out of which 14/18 (77.78%) positive in cases and 7/11 (63.64%) positive in control. (Table 1, 3)(Figure 2)

For HER–2/neu, it was positive in 21/29 (72.41%) overall, just like in EGFR mentioned above, but positive 12/18 (66.67%) in cases and 9/11 (81.82%) in control respectively. (Table 1, 3)(Figure 3)

Conclusion

Despite the efforts by many investigators, GBC continues to represent a major challenge in oncology. Surgical resection remains the only curative treatment for this disease. The roles of radiation, chemoradiation, and chemotherapy in neoadjuvant and adjuvant settings remained to be defined in prospective studies. With further studies based on molecular understanding and developing new targeted therapies, we will be in better position to manage GBC and increase the survival rate.

Key words

EGFR, HER–2/neu, GBC, Targeted therapy, chemotherapy, Immunohistochemistry

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الملخص

الخلفية

سرطان المرارة هو أكثر سرطانات السبيل الصفراوي خباثة شيوعا في الهند ، وعلى الرغم من التقدم الحديث في فهم بيولوجيا السرطان فما زال هذا المرض يمثل تحديا علاجيا ذو مآل سيئ وبقيا منخفضة .

وتمثل الجراحة الوسيلة العلاجية الأولية في حين تبقى الوسائل العلاجية الأخرى ذات طبيعة مساعدة في الأساس .وقد أجريت هذه الدراسة لتقييم تعبير مستقبلة عامل النمو البشروي EGFR ، ومستقبلة عامل النمو البشروي البشري 2 (HER -2/neu) في سرطانة المرارة.

الطريقة

هذه الدراسة الحالية كانت استباقية، وأجريت بمعهد عالي (ثالثي) فائق التخصص بشمال الهند ، وكانت دراسة رائدة وعند اكتمالها، كانت هناك 29 عينة مناسبة تم إخضاعها لاحقا لتقييم مستقبلة عامل النمو البشروي ومستقبلة عامل النمو البشروي البشري – 2

وشملت العينة كلا من حالات سرطانة المرارة عدد (18) ومرضى التهاب المرارة عدد (11) كمجموعة ضابطة . [جدول1.] بعد إجراء المعالجات الضرورية، فقد تم تحضين الشرائح مع الضد الأولي مستقبلة عامل النمو البشروي (EGFR) والجاهز للاستخدام (RTU) والتابع لشركة بيوجينكس ، الهند، وتخفيف HER-2/neu بمقدار 600:1 ، (داكوباتس) - الدانمرك . (وبعد ذلك ثم تحضين الشرائح مع الضد الثانوي) عتيدة كشف المنسوبة إلى ريل انفيسين ، داكوباتس ، الدانمرك.

وفي النهاية وعلى الشرائح الجاهزة (المحضنة) تم استخدام تلوين لغشاء الخلية لتقييم الإيجابية لكلا من مستقبلة عامل النمو البشروي ومستقبلة عامل النمو البشروي – 2 .

النتائج

كانت مستقبلة عامل النمو البشروي EGFR ايجابية في بصورة اجمالية في (72.41%) 21/29 بصورة إجمالية ، منهم (77.8%) 14/18 موجبا في الحالات ، و (63.64%) 7/11 موجبا في المجموعة الضابطة ، جدول 1 شكل 2

أما بالنسبة لمستقبلة عامل النمو البشروي البشري – 2 فقد كانت إيجابية في 21/29 (72.41%) بصورة إجمالية ، مثل ما ذكر سابقا في حال مستقبلة عامل النمو البشروي ، ولكنه كان إيجابيا في 12/18 (66.67) من الحالات ، و (81.82%) 9/11 في المجموعة الضابطة على التوالي . (جدول 1، 2) (الشكل 3) .

الخلاصة

على الرغم من جهود العديد من الباحثين، فإن سرطانة المرارة مازالت تمثل تحديا كبيراً في علم الأورام . ويبقى الاستئصال الجراحي هو الوسيلة العلاجية الشافية الوحيدة لهذا المرض .

ويبقى دور المعالجات الإشعاعية والكيميائية الإشعاعية والكيمائية محددا كعلاجات مساعدة أو كمساعدات حديثة كما تم ذكره في الدراسات الاستباقية . ومع مزيد من الدراسات التي تعتمد على الفهم الجزيئي وتطوير علاجات موجهة حديثة ، فسوف نكون بذلك قادرين بشكل أفضل على ايجاد تدابير علاجية لسرطانات المرارة وزيادة معدل البقيا .

الكلمات الرئيسية : سرطانة المرارة ، العلاج الموجه ، المعالجة الكيميائية ، الكيمياء الهيستونلوجية المناعية .

Background

GBC is the most common malignancy of the biliary tract in India. Despite the recent advancement in the understanding of the cancer biology, the disease still remains a therapeutic challenge with poor prognosis and low survival. High EGFR expression, tumor node metastasis (TNM) stage and preoperative serum carbohydrate antigen 19.9 (CA 19.9) were retained as independent predictors of overall survival. [1] Surgery plays a pivotal role in its management with radiotherapy and chemotherapy as an adjuvant setting. Understanding the molecular events in gallbladder carcinogenesis may provide novel targeted therapeutic approach. [2]

EGFR is a protein kinase receptor involved in the signal transduction affecting various cellular activities such as metabolism, transcription, cell cycle progression, apoptosis and differentiation. Studies from Asia and Australia have demonstrated EGFR overexpression in GBC. There is a trend toward shorter survival in patients (pts), having more poorly differentiated tumors with greater intensity of EGFR expression.

HER–2, also known as Neu, ErbB–2, CD340 (cluster of differentiation 340) or p185, is a protein that in humans is encoded by the ERBB2 gene. C–ErbB–2 protein is present mostly at surface epithelium in large and septal bile ducts but negative in hyperplasia or atypical hyperplastic bile duct epithelium which do not correlate with the histopathological grade or tumor stage. Overexpression of the gene product occurs in one fourth to about two thirds of carcinoma of biliary tract (BTC) and may be used as phenotypic marker for neoplastic transformation with a worse prognosis. [3] In one study it was found that GBC rarely overexpress HER–2/neu using the HercepTest [4] and no pt. co–expressed EGFR and HER–2/neu. [5] Genomic instability due to Myc (c–Myc) amplification may cause specific amplification of EGFR and/or ERBB2. Myc amplification is found only in 3.7% of the GBC cases but this information might be useful for tailored target therapy in the future. [6]

The p53 positivity increased with increasing grade while C–ErbB–2 positivity decreased with increasing grade of GBC. Mean age in C–ErbB–2 positive cases were lesser as compared to negative cases, while p53 did not show such association with age. C–ErbB–2 overexpression in adenoma and younger age group indicates its role as an early event in carcinogenesis of gallbladder. [7] Co–expression of p53 and C–ErbB–2 suggests that alterations of these genes might act in concert in the neoplastic transformation. [8]

With the recent success of EGFR targeted therapies in other solid tumors that over–expressed EGFR, it may now be appropriate to evaluate agents targeting this pathway either as single agent, or in combination with standard chemotherapy in GBC. However, k–ras mutation in pts with BTC (cholangiocarcinoma–intrahepatic–peripheral or hilar and extra–hepatic, GBC and ampullary carcinoma) warrants further investigations as use of EGFR inhibitors grows.

Methods

The present study is prospective and done in tertiary super–specialty institute of northern India. This is a pilot study, and at the time of completion 29 samples were submitted for EGFR and HER–2/neu evaluation including both cases of GBC and cholecystitis pts as control. (Table 1). Until date, all the gallbladder pts which were fit for this study were taken for the project. The inclusion criteria were adults, both sexes, all cases (metastatic, locally advanced or inoperable gallbladder), no other associated malignancies and no prior treatment in any form (surgery, chemotherapy and radiotherapy) with Karnofsky Performance Scale (KPS)> 50.

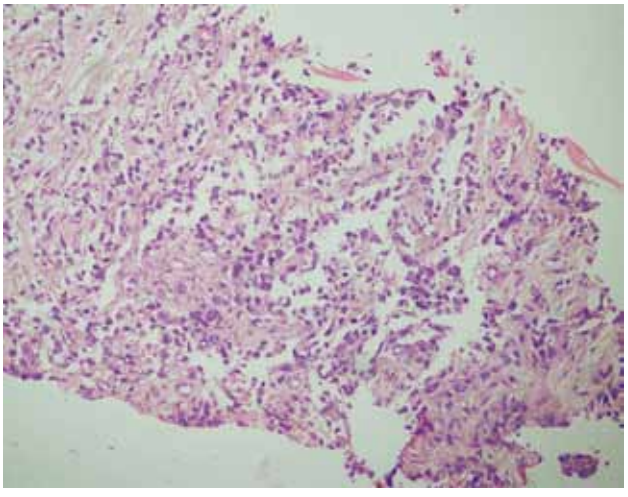

Figure 1: Adenocarcinoma gallbladder. H&E, 10X

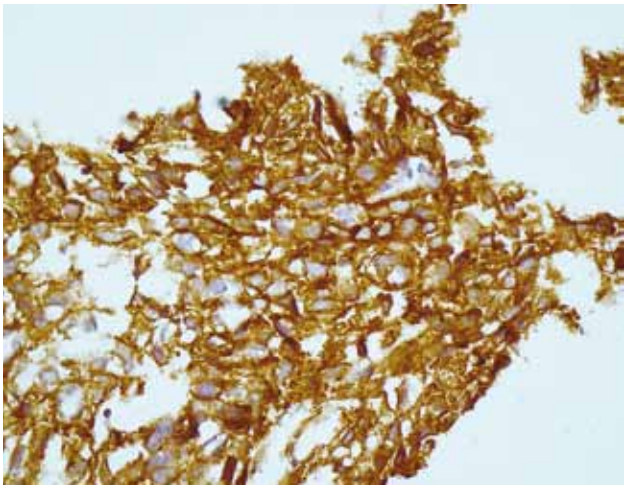

Figure 2: Adenocarcinoma gallbladder showing positive staining for EGFR (>90%, 3+). IHC, 40X

Patient (Total)	Adenocarcinoma (Cases)	Cholecystitis (Control)
29	18	11
Age(Years)	30–70	24–65
Median	48	50
Male	3	3
Female	15	8
EGFR Positive	77.78% (14/18)	63.64% (7/11)
HER–2/neu Positive	66.67% (12/18)	81.82% (9/11)

Table 1. Patient Characteristics

Intensity	Pattern of staining
0	no staining
1+	Distinct membrane staining visible at 40X magnification only
2+	Distinct membrane staining visible at 10X magnification only
3+	Distinct membrane staining visible at 5X magnification only

Table 2. Interpretation of intensity of EGFR and HER–2/neu immunohistochemistry

Assessment: After clinical and radiological assessment of the pt., tissue biopsy was done for confirmation of diagnosis. Bleeding, clotting and coagulation profile was done. Pt. was kept on empty stomach on the day of procedure. Consent of the pt. was taken after explaining the procedure and the complications related to it. At the time of procedure, it was mandatory that pt. was positioned appropriately, preferably supine to allow optimal condition for an adequate and safe biopsy. Under ultrasonographic guidance, examination performed with SA 800 (Medison) USG machines, having 3.5 MHz sector (mechanical) probe. Doppler imaging was also applied to the field to ensure the absence of large blood vessels in the plane. Once adequate imaging of the liver and gallbladder fossa has occurred, the biopsy was performed on all inoperable cases with 18 G Quick Core Cooks Biopsy Gun under aseptic technique and local anesthesia. At the time of biopsy, precaution was taken that the biopsy needle should pass in the same directional plane as the ultrasound transducer signal. Once the procedure was over, pt. was monitored for 1 hour prior to discharge for any complications. No complications were found in any pt. except mild pain at local site which was managed symptomatically.

The specimen was sent for tissue biopsy and was kept in tissue bank for EGFR and HER– 2/neu evaluation which was finally analyzed along with postoperative tissue specimen of operable cases of cholecystitis as control.

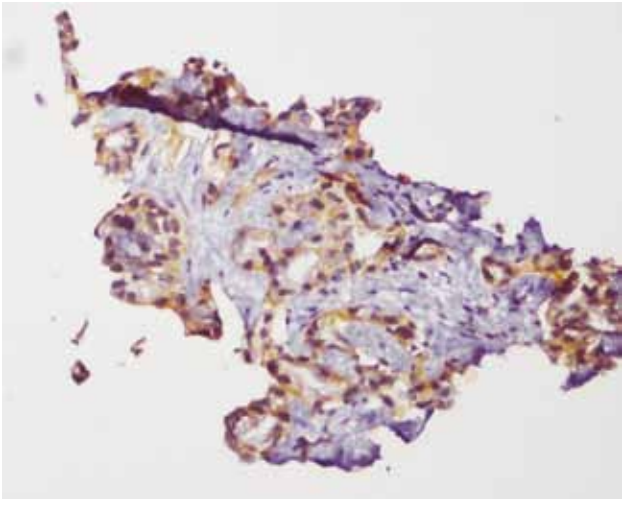

Figure 3: Adenocarcinoma gallbladder showing positive staining for HER– 2/ neu (70%, 2+). IHC, 20X

Authors	EGFR Positive (GBC)	EGFR Positive (Cholecystitis)	HER–2/ neu Positive (GBC)	HER–2/neu Positive (Cholecystitis)	References
Kamel D et al 1993			10.00%(3/10)	0.00% (0/4)	(8)
Lee CS et al 1995	100.00% (13/13)	64.00 (7/11)			(25)
Chow NH et al 1995			63.60% (7/11)	63.00% (29/46)	(3)
Zhou YM et al 2003	70.70% (41)	0.00% (0/10)			(13)
Matsuyama S et al 2004			11.70%		(4)
Nakazawa K et al 2005	8.10%		15.70%		(15)
Moon WS et al 2005	11.00%		62.00%		(23)
Chaube A et al 2006			25.00 (10)		(7)
Kawamoto T et al 2007			31.20%	12.50% (2+)	(9)
Kaufman M et al 2008	93.75% (15/16)				(5)
Ooi A et al 2009	11.34% (11/97)		14.43% (14/97)		(6)
Harder J et al 2009	60.71% (34/56)		41.13% (51/124)		(24)
Shafizadeh N et al 2010	80.00% (41/51)		4.00% (2/51)		(10)
Chang PY et al 2010	100.00 (3/3)				(19)
Pignochino Y et al 2010	38.5%		10.00%		(16)
Present study 2013	77.78% (14/18)	63.64% (7/11)	66.67% (12/18)	81.82% (9/11)	

Table 3. Comparison between different studies showing EGFR and HER–2/neu Positivity

Present study had 18 samples of cases, (14 Gun biopsy specimens and 4 open biopsy specimen in pts, who were found inoperable after laparotomy) and 11 post–cholecystectomy specimens not having malignancy, as control. Material adequate for immunohistochemical (IHC) studies was present in all 29 cases, and was subjected to IHC studies. Diagnosis of adenocarcinoma gallbladder was confirmed in 18 biopsy samples (Figure 1) and cholecystitis in 11 pts. (Table 1)

IHC Evaluation: In the pathology laboratory, serial 4 µ sections were cut from the tissue block and fixed overnight at 60ºc. Dewaxing was done by immersing the warm slides in xylene twice. After that slides were immersed in graded alcohol 100%, 70% and 50% and distilled water for 5 minutes. Blocking was done in 50 ml methanol and 1.5 ml H2O2 for 30 minutes and washed with distilled water for 5 minutes. Antigen retrieval was done in pre–warm citrate buffer (pH 6.0) at 90ºc for 90 minutes. Slides were cooled for 30 minutes at room temperature and washed 3 times in Tris–buffer (pH 7.5).

Slides were incubated in primary antibody EGFR ready to use (RTU) BioGenex, India and HER–2/neu dilution 1:600, Dakopatts (Denmark) overnight and washed three times in Tris–buffer (pH 7.5) again

followed by incubation with secondary antibody (Real Envision Detection Kit, Dakopatts, Denmark) for 30 minutes. After 3 washings of Tris–buffer (pH 7.5) slides were incubated in chromogen (DAB –3, 3'–diaminobenzidine tetra hydrochloride) buffer in 1:50 ratio followed by 3 washings of distilled water. Slides were then counterstained with haematoxylin followed by washing and air dried for 15–20 minutes. Finally the slides were mounted with DPX. Cell membrane staining was used to assess positivity for EGFR and HER 2/neu.

The interpretation of staining for EGFR and HER–2/neu was reported as percent positive cells and intensity of staining. For percent positive cells, total number of cells staining positive out of 100 cells was counted and an average of 10 high power fields was reported. The intensity of staining was interpreted as per Table 2.

Data analysis: We compared EGFR and HER–2/neu expression using of Fisher's exact test statistical method. We used the words 'two tailed' or 'two sided' for P value calculation. Analysis was done on MS Excel spreadsheet software. Significance was at P value of .05.

Results

The results of immuno–histochemical analysis of 29 pts of Gallbladder are adenocarcinoma 18/29 (62.07%) as cases (Figure 1) and cholecystitis 11/29 (37.93%) as control. For EGFR, it was overall positive in 21/29 (72.41%) pts, but positive in 14/18 (77.78%) in cases (Figure 2) and 7/11 (63.64%) in control respectively. (Table 1, 3)

- In positive cases (n=14), subset of expression was 1+ in 1/14 (7.14%), 2+ in 5/14 (35.71%) and 3+ in 8/14 (57.15%) while
- In positive control (n=7), subset of expression was 1+ in 5/7 (71.42%), 2+ in 1/7 (14.29%) and 3+ in 1/7 (14.29%)

When we applied the two tailed test between the cases and control for EGFR, p value=0.4327. The association between the above group and outcomes was considered to be not statistically significant. Likewise overall HER–2/neu was positive in 21/29 (72.41%) pts, out of which 12/18 (66.67%) positive in cases (Figure 3) and 9/11 (81.82%) positive in control. (Table 1, 3)

- In positive cases (n=12), subset of expression was 1+ in 0/12 (00.00%), 2+ in 5/12 (41.67%) and 3+ in 7/12 (58.33%).
- In positive control (n=9), subset of expression was 1+ in 1/9 (11.11%), 2+ in 5/9 (55.56%) and 3+ in 3/9 (33.33%).
- When we applied the two tailed test between the cases and control for HER–2/neu, p value=0.6706. The association between the above group and outcomes was considered to be not statistically significant.
- In 17/29, (58.62%) of pts, sample express both EGFR and HER–2/neu together, out of which 10/17, (58.82%) were cases and 7/17, (41.18%) were control. The two–tailed P value equals 0.7513. The association between groups and outcomes was considered to be not statistically significant.
- In 4/29, (13.79%) of pts sample didn't express neither EGFR nor HER–2/neu out of which 2/4, (50.00%) are cases and 2/4, (50.00%) are control. The two–tailed P value equals 1.0000. The association between groups and outcomes is considered to be not statistically significant.

Review

BTCs are uncommon in the United States but are endemic in South America and Asia. These are aggressive tumors associated with poor prognosis and survival. Standard chemotherapy often offers minimal benefit. Activation of HER–2/neu and/or EGFR are important in breast, colon, and lung cancer with exploration of its role in BTCs has been going on. [9] HER–2/neu expression is uncommon in these tumors [10] but our study did not support this version. HER–2/neu activates the Akt/NF–kappa B anti–apoptotic cascade to confer resistance to Tumor Necrosis Factor (TNF) on cancer cells which ultimately reduce host defense against neoplasia. [11]

EGFR signaling has recently been implicated in the genesis and progression of cholangiocarcinomas. EGFR mutations were more common in intra–hepatic or poorly differentiated tumors. Differences in age, sex, stage at diagnosis and survival was not observed between mutation positive and negative pts. Therefore a highly selected application of the EGFR kinase inhibitor would be therapeutically effective in these pts. [12]

Cholelithiasis and cholecystitis were closely related to GBC. The positive expression rates of epidermal growth factor (EGF) and EGFR were higher in GBC and dysplasia than in simple hyperplasia and normal gallbladder tissue. Proliferating cell nuclear antigen (PCNA) counting score tended to increase with the severity of mucosa change degree. Significant positive correlation was shown between EGF, EGFR and PCNA. [13]

There is increasing evidence that overexpression of tyrosine kinase growth factor receptors such as ErbB–2, EGFR and Met may play important roles in the development of BTC. Increased ErbB–2/EGFR heterodimer formation, activation of mitogen–activated protein kinase, and up–regulation of cyclooxygenase–2 level (mRNA and protein) were observed in gallbladder epithelium. [14] It is proposed that the new adjuvant chemotherapies could be directed to BTC in which ErbB–2 and EGFR are overexpressed. [15]

EGI–1 cell line is highly sensitive to gemcitabine and combination with gefitinib further increases the anti–proliferative effect of gemcitabine. In TFK1 and TGBC1–TKB cells, the efficacy of gemcitabine is increased with addiction of sorafenib and everolimus. In TGBC1–TKB cells, lapatinib also has a synergic effect with gemcitabine. HuH28 becomes responsive if treated in combination with erlotinib and sensitive to lapatinib as a single agent. [16]

In one case report, complete response in GBC to erlotinib plus gemcitabine, which does not require mutation of EGFR gene (wild type genotype). [17] Presence of k–ras mutation may alter erlotinib efficacy, the combination of bevacizumab and erlotinib may be a therapeutic alternative in pts with advanced BTC.[18] Cetuximab containing therapy might be an effective treatment for advanced BTC in the pts who have wild type k–ras. [19]

The recent phase III study showed longer survival with gemcitabine and cisplatin than gemcitabine alone. Lapatinib is an oral inhibitor of EGFR and Her2/neu with evidence of activity in a number of tumor types. In a case report, dramatic response has been quoted to Panitumumab and Bevacizumab in widespread metastatic GBC. [20]

Further understanding of the molecular mechanism of carcinogenesis coupled with more extensive genetic profiling of GBC pts will help to assess the therapeutic relevance of targeting a specific pathway. [21]

TGF–alpha overexpression is present in most GBC but with no significant differences in expression between different tumor grades. None of the cases of gallbladder dysplasia or chronic cholecystitis had strong TGF–alpha expression and this was significantly different from the carcinomas. No statistically significant correlation between TGF–alpha expression with pt. survival or tumor recurrence was found. Correlation was found between TGF–alpha and EGFR expression in gallbladder and biliary tract tumors. [22]

Conclusion

Although anatomically BTCs are related and have similar metastatic patterns, each has a distinct clinical presentation, molecular pathology and prognosis. Surgical resection remains the only curative treatment for this disease. The roles of radiation, chemoradiation, and chemotherapy in neoadjuvant and adjuvant settings remained to be defined in prospective studies. Further understanding of the molecular events in gallbladder carcinogenesis may provide new novel targeted therapeutic approaches as well as prediction of getting cancer in cholecystectomy pts, but needs more robust research in this regard.

Acknowledgement

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