

Expression of Her-2/neu in extrahepatic cholangiocarcinoma

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Background: Receptor tyrosine-protein kinase erbB-2, which is also frequently called human epidermal growth factor receptor-2 (Her-2) or Her-2/neu, has been found to be overexpressed in various human cancers.

Hypothesis: The aim of this pilot study was to explore the frequency of Her-2/neu gene amplification and protein expression in extrahepatic cholangiocarcinoma (EHBC). We used the World Health Organization classification criteria for EHBC.

Materials and methods: This was a retrospective study using 88 tissue samples, including 45 samples from non-neoplastic biliary tissue (NNB) and 43 samples of extrahepatic cholangiocarcinoma (EHBC). A tissue microarray including NNB and EHBC was constructed and analyzed by immunohistochemistry (IHC) and dual in situ hybridization for Her-2/neu protein expression and amplification, respectively. The Her-2/neu expression was scored following the guidelines used for the ToGA study.

Results: All NNB samples and all but one EHBC samples showed no expression of Her-2/neu by IHC. The one EHBC case immunohistochemically positive for Her-2/neu had an IHC score of 3+. Her-2/neu gene amplification was present in two EHBC samples only and included the case found to be positive by IHC.

Conclusion: Our findings are similar to those reported in the literature. Although Her-2/neu overexpression has been documented in many types of cancer, Her-2/neu protein overexpression tends to have no role in the development and/or progression of EHBC.

Keywords: extrahepatic, cholangiocarcinoma, Her-2/neu, ToGA, immunohistochemistry

Introduction

Extrahepatic cholangiocarcinoma (EHBC) is the second most common biliary tract malignancy^{1,2} with an incidence in the US being 1.2 per 100,000 men and 0.8 per 100,000 women.³ EHBC usually affects patients over 65 years of age, with slightly higher rates of whites affected compared to blacks.^{2,3}

While most of the EHBCs occur in an otherwise normal liver, 10% of them are predisposed by chronic inflammatory conditions such as primary sclerosing cholangitis, infestations with liver flukes, choledochal cysts, and hepatic biliary stones.^{3,4} Intraductal papillary neoplasms and biliary intraepithelial neoplasia have been proposed in the latest World Health Organization classification as precursor lesions for the development of EHBC.¹

Many molecular factors have been related to the pathogenesis of EHBC, such as mutation of the *K-ras*, *p53*, and *APC* genes, as well as the loss of heterozygosity at the *p53* and *APC* gene loci.⁵ In addition, it has been shown that the methylation profile of

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EHBC often includes the methylation of tumor suppressor genes such as the *RASSF1A*, *p15*, *p16*, *APC*, *E-cad*, *MGMT*, *GSTP*, *RAR-β*, and *DAPK*.⁶

Human epidermal growth factor receptor-2 (Her-2/neu) is a tyrosine kinase receptor⁷ that stimulates numerous pathways regulating cell proliferation, differentiation, and apoptosis and has been overexpressed and amplified in various cancers.⁷ The amplification and overexpression of the Her-2/neu proto-oncogene has been described in various malignancies such as breast and ovarian cancer⁸ as well as in gastric cancer.^{9,10}

The aim of this study is to investigate the frequency of Her-2/neu overexpression using immunohistochemistry (IHC) and the frequency of Her-2/neu amplification using dual in situ hybridization (DISH) in EHBC.

Materials and methods

Upon Institutional Review Board approval the histological specimens and reports from 43 patients who underwent resection for EHBC were re-evaluated by two pathologists with interest in gastrointestinal pathology (MG and DC) to confirm the diagnosis for inclusion in the current Her-2/neu study. The patients signed a written informed consent to be part of this study.

Clinicopathologic characteristics

The patients' ages ranged between 41 and 89 years (mean 66.5 years). Twenty-three of them were female and 20 were male. The tumor ranged in size between 0.5 and 3.7 cm (mean 1.9 cm). All tumors were extrahepatic adenocarcinomas, intestinal type (World Health Organization classification). Nine tumors were well differentiated, 25 were moderately differentiated, and 9 were poorly differentiated. The clinicopathologic features of the tumors are summarized in Table 1.

Tissue microarrays

The hematoxylin and eosin stained slides of 43 EHBC patients were selected and the area of tumor was marked. The paraffin block corresponding to the selected slide was used to construct an EHBC tissue microarray (EHBC-TMA). All tissues had been processed for 12 hours in 10% neutral buffered formalin, a series of graded alcohols, xylene, and paraffin. After processing, each tissue was embedded in histology grade paraffin and then cut on a Leica microtome. A section was picked up on a glass slide and stained with hematoxylin and eosin, and a coverslip was placed on the slide for permanent mounting.

The EHBC-TMA also comprised samples on non-neoplastic biliary tissue (NNB). Three 1 mm cores from each of

the formalin-fixed, paraffin-embedded samples were included in the EHBC-TMA. The NNB samples were collected from the same EHBC patients near the uninvolved surgical margin and away from the tumor. In 2 cases, the NNB samples were taken from specimens resected for non-neoplastic conditions.

Immunohistochemistry

The usage of EHBC-TMA in our study allowed the entire cohort, NNB and EHBC tissues, to be analyzed on one slide, and the benefits of using TMA are well documented.¹¹ A known Her-2/neu-positive breast carcinoma was used as a positive control. For negative control, the primary antibody was omitted during the incubation step. The EHBC-TMA was incubated for 16 min with a Her-2/neu rabbit monoclonal antibody (clone 4B5, using the Benchmark XT; Ventana Medical Systems) and detected by ultraview universal DAB detection kit (using the Benchmark XT; Ventana Medical Systems, Inc.). Antigen retrieval was performed using the Ventana Cell Conditioner #1 for 32 min. The Her-2/neu protein expression was evaluated using the modified ToGA trial scoring criteria established for gastric and gastroesophageal carcinomas¹²⁻¹⁴ as follows: 1) no staining or no membranous staining of tumor cells was scored as "0"; 2) tumor cells with faint membrane staining irrespective of the percentage of tumor cells were scored as "1+"; 3) tumor cells with weak to moderate membrane staining irrespective of the percentage of tumor cells were scored as "2+"; and 4) tumor cells with strong complete, basolateral, or lateral membrane reactivity irrespective of the percentage of tumor cells were scored as "3+".¹⁴ We used the presence of complete, basolateral, or lateral membranous reactivity in $\geq 10\%$ of cells as the cutoff for positivity, as required by the published guidelines.¹⁴ The Her-2/neu IHC score was calculated by two independent pathologists (RS and DC) and a consensus score was reached for each specimen.

Dual in situ hybridization

The Her-2/neu gene amplification by in situ hybridization was performed using the Ventana INFORM HER2 Dual ISH DNA Probe Cocktail assay (Ventana Benchmark Ultra Platform). Analysis of in situ hybridization was performed using a 60× objective. A manual count of the number of Her-2/neu signals and the number of centromere 17 (CEP17) signals in each individual nucleus was performed using bright field microscopy and a minimum of 20 epithelial cells were evaluated per sample. The ratio of the average number of Her-2/neu gene copies to the average number of Chr17 copies was calculated manually.

Indeterminate results were obtained due to any of the following: absence of target cells, no tissue present, unable to

Table 1 Patients' clinicopathologic characteristics

Age at diagnosis (years)	Gender	Diagnosis	Tumor size (cm)	Tumor grade	Tumor stage
48	F	Cholangiocarcinoma	1.5	Moderately differentiated	pT2N0MX
50	F	Cholangiocarcinoma	1.7	Moderately differentiated	pT1N0M0
56	F	Cholangiocarcinoma	3.0	Moderately differentiated	pT1N0M0
57	F	Cholangiocarcinoma	2.0	Well differentiated	pT1N0M0
57	F	Cholangiocarcinoma	2.0	Moderately differentiated	pT3N0MX
58	F	Cholangiocarcinoma	3.0	Well differentiated	pT3N0MX
60	F	Cholangiocarcinoma	1.2	Moderately differentiated	pT3N1MX
61	F	Cholangiocarcinoma	1.7	Moderately differentiated	pT1N0MX
61	F	Cholangiocarcinoma	1.5	Moderately differentiated	pT2N1MX
62	F	Cholangiocarcinoma	3.5	Poorly differentiated	pT3N1MX
62	F	Cholangiocarcinoma	0.5	Well differentiated	pT3N0MX
63	F	Cholangiocarcinoma	3.1	Moderately differentiated	pT2N0MX
66	F	Cholangiocarcinoma	1.7	Well differentiated	pT3N1MX
66	F	Cholangiocarcinoma	2.0	Moderately differentiated	pT3N0MX
66	F	Cholangiocarcinoma	1.7	Poorly differentiated	pT1N1MX
69	F	Cholangiocarcinoma	1.5	Poorly differentiated	pT1N1MX
75	F	Cholangiocarcinoma	2.2	Moderately differentiated	pT3N1MX
77	F	Cholangiocarcinoma	2.0	Poorly differentiated	pT3N1MX
78	F	Cholangiocarcinoma	2.4	Moderately differentiated	pT3N1MX
82	F	Cholangiocarcinoma	1.8	Poorly differentiated	pT3N1MX
89	F	Cholangiocarcinoma	1.8	Moderately differentiated	pT3N0MX
67	F	Cholangiocarcinoma	1.5	Moderately differentiated	pT3N0MX
41	M	Cholangiocarcinoma	1.7	Poorly differentiated	pTxNxM1
58	M	Cholangiocarcinoma	1.4	Poorly differentiated	pT3N0MX
58	M	Cholangiocarcinoma	1.3	Poorly differentiated	pT2N1MX
59	M	Cholangiocarcinoma	2.5	Moderately differentiated	pT2N0MX
61	M	Cholangiocarcinoma	1.8	Well differentiated	pT3N1MX
61	M	Cholangiocarcinoma	3.0	Moderately differentiated	pT3N1MX
64	M	Cholangiocarcinoma	2.0	Poorly differentiated	pT2N1MX
65	M	Cholangiocarcinoma	2.2	Well differentiated	pT1N0MX
66	M	Cholangiocarcinoma	1.0	Moderately differentiated	pT2N0MX
68	M	Cholangiocarcinoma	2.0	Well differentiated	pT2N0MX
68	M	Cholangiocarcinoma	2.5	Well differentiated	pT3N1MX
70	M	Cholangiocarcinoma	1.5	Moderately differentiated	pT1N1MX
70	M	Cholangiocarcinoma	1.3	Moderately differentiated	pT2N0MX
73	M	Cholangiocarcinoma	2.5	Moderately differentiated	pT2N0MX
73	M	Cholangiocarcinoma	1.5	Moderately differentiated	pT3N0MX
76	M	Cholangiocarcinoma	1.6	Moderately differentiated	pT2N0MX
79	M	Cholangiocarcinoma	0.5	Well differentiated	pT4N0MX
80	M	Cholangiocarcinoma	1.5	Moderately differentiated	pT3N1MX
83	M	Cholangiocarcinoma	0.7	Moderately differentiated	pT2N0MX
84	M	Cholangiocarcinoma	3.7	Moderately differentiated	pT3N0MX
74	F	Cholangiocarcinoma	1.9	Moderately differentiated	pT3N1MX

Abbreviations: F, female; M, male.

distinguish normal cells from target cells, unacceptable background or weak/absent ISH staining in target cells. A Her-2/neu:Chr17 ratio of <2.0 was considered DISH negative, while a Her-2/neu:Chr17 ratio ≥ 2.0 was considered DISH positive.

Results

The characteristics of the 43 subjects, including age, gender, pathological diagnosis, tumor size, tumor grade, and tumor stage, are shown in Table 1.

All NNB samples were negative for Her-2/neu IHC (score 0). We found all but one of the 43 EHBC cases to be negative for Her-2/neu expression. The positive case had an IHC score of 3+ (Figure 1A, B). Importantly, the Her-2/neu IHC score was similar for all of the 3 cores taken from this tumor. The scoring was based on the evaluation of the epithelial cells only.

The positive case was a moderately differentiated adenocarcinoma of the bile duct. Neither lymphovascular invasion nor distant metastasis was detected. This tumor had no

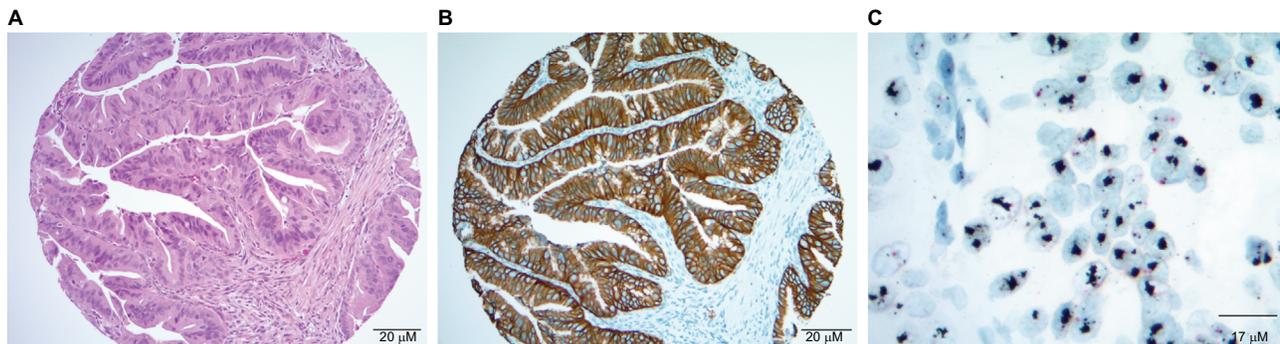


Figure 1 Extrahepatic Cholangiocarcinoma and Her-2/neu testing.

Notes: (A) A well-differentiated to moderately differentiated adenocarcinoma of the bile duct showing an invasive tumor with papillary morphology and associated desmoplastic stroma. The tumor cells have pink cytoplasm and elongated to polygonal nuclei with anisocytosis and loss of polarity (hematoxylin and eosin, 40 \times). (B) The adenocarcinoma shown in Figure 1A is strongly and diffusely positive for Her-2/neu (3+) and exhibits a well-defined membranous pattern of stain (40 \times). (C) Dual in situ hybridization demonstrating amplification of human epidermal growth factor receptor-2 in a moderately differentiated adenocarcinoma.

evidence of metastatic disease to the lymph nodes or distant organs (T₁N₀Mx).

The entire TMA was evaluated using DISH as part of this study, regardless of the IHC results. The DISH results demonstrated Her-2/neu amplification (ratio >2) in 2 cases (6 cores). One of these Her-2/neu-positive cases was the one that was found to be positive by IHC (Figure 1C).

Discussion

In the current study, we examined Her-2/neu protein expression in EHBC and found only 1 out of 43 cases (2.3%) to overexpress Her-2/neu. All the NNB samples were Her-2/neu negative.

Our results are in agreement with a few other reported studies investigating Her-2/neu protein expression in EHBC.

In one of these studies, Shafizadeh et al showed Her-2/neu expression in 2 out of 19 (4%) EHBC cases. These positive cases had a moderate and diffuse Her-2/neu staining (IHC score 2). The patients with positive Her-2/neu tumors were reported to have tumors of high grade and high stage upon presentation.¹⁵ This result is in disagreement with our finding where the only Her-2/neu-positive tumor was a moderately differentiated adenocarcinoma stage 1.

Yoshikawa et al reported their analysis of a larger group of EHBC patients where they observed the expression of Her-2/neu in 11 out of 130 (8.5%) EHBC patients. These authors described the Her-2/neu expression in well-differentiated and moderately differentiated tumors.¹⁶

The original study by Nakazawa et al compared the Her-2/neu expression in EHBC and in gallbladder carcinomas. They found significant expression of Her-2/neu in 14 out of 89 (15.7%) ($P < 0.05$) gall bladder carcinomas, compared to only 4 out of 78 (5%) EHBC.¹⁷ All (100%) 3+ Her-2/neu and 67% of 2+ Her-2/neu tumors showed Her-2/neu gene amplification

as well. These authors speculated that Her-2/neu protein is expressed in early EHBC disease and it is subsequently lost in late advanced EHBC disease.¹⁷

Other studies have reported higher percentage of Her-2/neu protein expression in EHBC. For example, Kim et al showed Her-2/neu protein expression in 16 out of 55 cases (29%) of EHBC with an IHC score of either 2 or 3.¹⁸ These authors also detected Her-2/neu gene amplification in 10 out of 55 patients (18%). Although there was no significant correlation between HER-2/neu protein expression and gender, age, location, tumor size, histologic grade, TNM Classification of Malignant Tumors stage, and presence or absence of lymphatic or perineural invasion, HER-2/neu protein overexpression tended to have a higher rate of distant metastasis.¹⁸

Zheng and Zhu reported an even higher percentage (60 of 75 cases; 80%) of EHBC with Her-2/neu overexpression. Her-2/neu expression in EHBC was commonly seen in high histological grade tumors compared to grade I tumors ($P < 0.05$). Perineural invasion and distant metastasis was significantly ($P < 0.05$) related to Her-2/neu-positive tumors.¹⁹

Expression of Her-2/neu, a tyrosine kinase receptor, in cancer has a great clinical significance due to the fact that this receptor can be targeted pharmaceutically. Numerous trials in breast cancer,^{20,21} stomach cancer,^{12,13,22} and bladder cancer^{23,24} have shown the therapeutic benefit of targeting Her-2/neu with adjuvant therapy, especially in the metastatic setting.

Reports of isolated cases of patients with Her-2/neu-positive metastatic gall bladder and pancreatobiliary tumors effectively responding to adjuvant therapy^{25,26} have raised the hope that EHBC could be therapeutically targeted. However, our results did not show a high percentage of Her-2/neu-positive EHBC. The discrepancy with earlier reports, showing a higher percentage of Her-2/neu-positive EHBC, may be due to the different type of antibody used (Table 2), or

Table 2 Her-2/neu antibodies used in the current study compared to others

Author	Method	Antibody source
Kim et al (2007) ¹⁸	Manual	Zymed lab, South San Francisco, CA, USA
Yoshikawa et al (2008) ¹⁶	Automated (polymer-based method, EnVision + Dual Link System-HRP)	Dako Denmark A/S, Glostrup, Denmark
Zheng and Zhu (2007) ¹⁹	Manual	Dako Denmark A/S, Glostrup, Denmark
Nakazawa et al (2005) ¹⁷	Manual	Nichirei, Tokyo, Japan
Shamekh et al (present study)	Automated (Ventana Medical Systems, using the Benchmark XT)	Ventana Medical Systems, Inc. Tucson, AZ, USA

Abbreviations: Her-2, human epidermal growth factor receptor-2; HRP, horseradish peroxidase.

to the preanalytical factors such as warm and cold ischemic time, type of fixative, time of fixation, and so on. Some of the previous studies were conducted before the publication of the College of American Pathologists guidelines for Her-2/neu testing,¹⁴ and their authors may have used different tissue preparation and IHC scoring criteria.

In conclusion, we found that Her-2/neu expression in EHBC is a rare event. Our finding is in agreement with a portion of the current literature on this topic.

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Disclosure

The authors report no conflicts of interest in this work.

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