Gene amplification and protein overexpression of HER-2/neu in human extrahepatic cholangiocarcinoma as detected by chromogenic in situ hybridization and immunohistochemistry: its prognostic implication in node-positive patients


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Background: In cholangiocarcinoma (CC), HER-2/neu protein overexpression has rarely been reported and the results are conflicting. The present study aimed to clarify the rates of HER-2/neu protein overexpression and gene amplification in human extrahepatic CC and to evaluate the correlation between HER-2/neu and several clinicopathologic features.

Patients and methods: We investigated HER-2 gene amplification by chromogenic in situ hybridization (CISH) and HER-2/neu protein overexpression by immunohistochemistry in 55 extrahepatic CC patients who underwent curative surgery at our institution.

Results: Overexpression of HER-2/neu protein (staining intensity score ≥ 2) was found in 16 out of 55 patients (29.1%). CISH revealed that HER-2 gene signals were increased in 10 out of 55 patients (18.1%). There was a positive and significant correlation between HER-2 gene amplification and HER-2/neu protein overexpression (Spearman’s rho = 0.718, P < 0.01). In subgroup with lymph node metastases, HER-2 gene amplification by CISH was significant prognostic factor for survival (OR 43.6, 95% confidence interval 1.6–1219.6).

Conclusions: HER-2/neu protein overexpression by HER-2 gene amplification may occur in human extrahepatic CC and constitute an independent prognostic factor in patients with lymph node metastases. In subgroup with lymph node metastases who exhibit HER-2/neu overexpression might constitute potential candidates for new adjuvant therapy, such as humanized monoclonal antibodies.

Key words: chromogenic in situ hybridization, extrahepatic cholangiocarcinoma, HER-2/neu, immunohistochemistry
HER-2/neu overexpression are used as both prognostic and predictive markers for breast cancer. As a prognostic marker, HER-2/neu is used to predict the probable course and outcome of the disease. As a predictive marker, HER-2/neu is used to forecast the patient’s therapeutic response to adjuvant chemotherapy and endocrine therapy and to select patients for anti-HER-2/neu monoclonal antibody (Trastuzumab) immunotherapy. In these patients, treatment with trastuzumab has been determined to reduce the volume of tumors, to augment chemotherapeutic effects, and to enhance survival rates in both primary and metastatic breast cancer patients [11]. Favorable clinical results with anti-HER-2/neu antibodies in breast cancer have led to the analysis of HER-2/neu expression in other solid tumors. HER-2 amplification and/or HER-2/neu overexpression have also been detected in ovarian [12], lung [13], gastric [14], and colon carcinomas [15]. HER-2/neu overexpression has been associated with poor prognosis in several tumor types as well [16]. Trastuzumab is currently on trial for other epithelial malignancies.

In CC, HER-2/neu protein expression has rarely been reported [17–22] and additionally, the results of HER-2/neu protein overexpression in those studies are conflicting. While 27%–82% of tumors were found to express HER-2/neu in CC of Thai, English, Taiwanese, American, and Japanese origin [18–22], Collier et al. [17] were unable to detect the expression of membranous HER-2/neu in 10 cases of human CCs of European Caucasian origin by immunohistochemistry. In Japanese study [22], it was indicated that positive immunohistochemical (IHC) staining of HER-2/neu in CC correlated with a shorter survival. However, definite correlation between HER-2/neu protein overexpression by HER-2 gene amplification and patient’s outcome in CC has not been clarified as yet.

In this study, we aimed to clarify the expression rates of HER-2/neu protein overexpression and HER-2 gene amplification in human extrahepatic CC and to evaluate the correlation between the expression status of HER-2/neu and several clinicopathologic features, including survival of human extrahepatic CC.

patients and methods

patients and tissue specimens

A total of 35 extrahepatic CC patients who underwent curative surgery from January 1994 to November 2004 at Kangbuk Samsung Hospital were included in this study. Patients included 34 men and 21 women with an average age of 60 years (range 27–77). Sixteen patients (29.1%) had history of cholelithiasis or choledocholithiasis. The histology was well-differentiated adenocarcinoma in 15 (23.6%), moderately differentiated adenocarcinoma in 32 (58.2%), and poorly differentiated adenocarcinoma in nine (16.4%) patients. The location of primary tumor was classified as hilar or nonhilar according to the Bismuth et al. [23] in which hilar CC was defined as a tumor involving the primary ductal confluence with or without extension into more proximal bile ducts. In the current study, there were 16 (29.0%) patients with hilar CC and 39 (71.0%) with nonhilar tumors. All of the cancers were staged following surgery and pathologic examination of the resected specimen using the tumor–node–metastasis (TNM) staging system [24].

Each tissue was fixed in 10% neutral buffered formalin, embedded in paraffin and verified by a histopathologist before including in our study. Our study was approved by the Institutional Review Board of Kangbuk Samsung Hospital.

IHC staining

IHC staining for HER-2/neu was conducted on 5 μm-thick sections, which were obtained from routine tissue blocks. In brief, after deparaffinization in xylene, the slides were washed with phosphate-buffered saline. Endogenous peroxidase activity was quenched by a 15-min incubation in methanol with 3% hydrogen peroxide (Sigma Chemical Co., Deisenhofen, Germany). Nonspecific binding was blocked by the application of normal rabbit serum in a humidity chamber at a 1:10 dilution, for 30 min. The slides were blootted, and the primary polyclonal rabbit antibody against human HER-2/neu protein (Zymed lab, South San Francisco, CA) was applied for 45 min at room temperature. Secondary goat anti-rabbit antibody (Zymed lab) linked to horseradish peroxidase was applied for 1 h at room temperature. The bound antibody was visualized using a peroxidase chromogen substrate. The sections were then counterstained with hematoxylin, and coverslipped. The four-tiered scoring system suggested by the manufacturer for use in breast cancer was utilized. Undetectable staining or membrane staining in <10% of the tumor cells was defined as a score of zero. Score 1+ was defined as faint membrane staining in >10% of the tumor cells; 2+ was defined as weak to moderate complete membrane staining in >10% of the tumor cells; and 3+ was defined as moderate to strong complete membrane staining in >10% of the tumor cells. HER-2/neu protein overexpression was defined as either negative (score 0 or 1+) or positive (score 2+ and 3+).

This cut-off point was predicted on the results of previous breast cancer studies. Interpretations were made independently by two pathologists, who had been blinded to each other’s findings, and to the results of the other assays. Negative control staining was conducted either by omission of the primary antibody, by the use of non-immune serum and irrelevant antibodies, or by preincubation of primary antibodies with the peptide antigen (1:10; Oncogene Science, 80 Rogers Street, Cambridge, MA 02142 USA). We used paraffin slides of invasive breast carcinoma as a positive control.

chromogenic in situ hybridization

Chromogenic in situ hybridization (CISH) analysis was applied to all 2+ and 3+ tumors, as well as to all immunohistochemically negative tumors (0 and 1+). We used paraffin slides of invasive breast carcinoma as a positive control. CISH was conducted with the Zymed SpotLight® HER2CISH™ Kit (Zymed lab) according to the manufacturer’s instructions. The formalin-fixed, paraffin-embedded, 5-μm tissue slides were evaluated for the HER-2 gene copy number, using conventional peroxidase reactions under the brightfield microscope (×40), The Zymed SpotLight® HER2CISH™ Kit uses a double-stranded DNA probe that has been labeled with digoxigenin. This HER-2 probe specifically bind to the HER-2 gene locus on chromosome 17q12-21. An individual HER-2 gene appears as a small round single dot. Tumors with no amplification of HER-2 show typically one to two dots per nucleus, or three to five dots in polysony cases. HER-2 gene amplification is typically visible as DAB-stained large clusters, multiple individual dots, mixed large clusters, and multiple individual dots or small clusters. Stromal and inflammatory cells were excluded from analysis, on the basis of the morphological features of their nuclei.

results

The mean (± standard deviation) postoperative follow-up period was 26.0 (± 27.7) months (median 17, range 2–130 months).

overexpression of HER-2/neu by immunohistochemistry

Immunohistochemistry showed that HER-2/neu protein was expressed in the cell membrane of cancer cells (Figure 1). Stromal cells and normal epithelial cells adjacent to the tumor tissue were negative. Thirty-six (65.5%) of the 55 investigated
extrahepatic CC were classified as score 0, three (5.4%) were classified as score 1+, 14 (25.5%) were classified as score 2+, and two (3.6%) were classified as score 3+. Positive immunostaining (2+ or 3+) for HER-2/neu protein was detected in 16 (29.1%) out of 55 extrahepatic CCs analyzed.

The clinicopathologic characteristics of 55 extrahepatic CC patients according to the presence or absence of HER-2/neu protein overexpression are summarized in Table 1. Tumors with HER-2/neu protein overexpression showed the tendency of higher rate of distant metastasis (18.8% versus 2.6%, \( P = 0.06 \)). There was, however, no significant correlation between HER-2/neu protein overexpression and age, gender, tumor size, location, TNM stage, histologic differentiation, and presence or absence of lymphatic or perineural invasion in patients with extrahepatic CCs.

**HER-2 gene amplification by CISH**

In CISH analyses, gene amplification was detected in 10 (18.1%) out of 55 patients with extrahepatic CC. When the results of CISH and IHC were compared, eight tumors (57.1%) among the 14 tumors with 2+ immunostaining showed gene amplification, and all of two tumors with 3+ immunostaining also showed gene amplification by CISH. There was a positive and significant correlation between HER-2 gene amplification and HER-2/neu protein overexpression (Spearman’s rho = 0.718, \( P < 0.01 \)). Among other tumors which showed 0 or 1+ immunostaining, none showed gene amplification by CISH. Large clusters or mixture of multiple individual dots (>10 dots) and large clusters of the HER-2 gene present per nucleus in >50% tumor cells were noticed in two tumor specimens. Signals of six to 10 individual dots, or small clusters of the HER-2 gene present per nucleus in >50% tumor cells were noticed in the remaining eight tumor specimens (Figure 2).

**survival analyses**

In univariate analyses, patients with older age (>58 years), larger size of tumor (≥3.5 cm), advanced TNM stage, and presence of lymphatic or perineural invasion showed poor survival (Figure 3). The status of HER-2/neu protein overexpression and HER-2 gene amplification did not have impact on the total patient’s survival. However, for patients with lymph node metastases (\( n = 24 \)), HER-2 gene amplification was poor prognostic factor for survival by univariate analysis (Figure 4).

| Table 1. The clinicopathological characteristics of 55 extrahepatic cholangiocarcinoma patients according to the presence or absence of HER-2/neu protein overexpression |
| Clinicopathologic characteristics | HER-2/neu overexpression positive \((n = 16)\) | HER-2/neu overexpression negative \((n = 39)\) | \( P \) value |
| Age (years, mean ± SD) | 57.9 ± 12.0 | 61.3 ± 8.5 | NS |
| Gender (male : female) | 11 : 5 | 23 : 16 | NS |
| Tumor size (cm, mean ± SD) | 3.1 ± 1.3 | 2.9 ± 1.8 | NS |
| Location (hilar/nonhilar) | 6/10 | 10/29 | NS |
| T stage (1/2/3/4) | 2/3/9/2 | 4/16/14/5 | NS |
| N stage (0/1) | 10/6 | 21/18 | NS |
| M stage (0/1) | 13/3 | 38/1 | 0.06 |
| TNM stage (IA/IB/IIA/IIIB/IV) | 2/2/5/4/0/3 | 4/9/7/15/3/1 | NS |
| Histologic differentiation | | | |
| Well differentiated | 2 | 11 | |
| Moderately differentiated | 11 | 21 | |
| Poorly differentiated | 3 | 6 | |
| Lymphatic invasion (+) | 9 (56.3%) | 22 (56.4%) | NS |
| Perineural invasion (+) | 9 (56.3%) | 20 (51.3%) | NS |

SD, standard deviation; NS, not significant; TNM, tumor–node–metastasis.

**Cox regression analyses**

A Cox proportional hazards model identified TNM stage, older age (>58 years), and poorly differentiated histology as poor prognostic factors in total patients group (Table 2). As for the patients with lymph node metastases, larger size of tumor (≥3.5 cm), hilar location, poorly differentiated histology, and the presence of HER-2 gene amplification by CISH were independent poor prognostic factors (Table 3).

**discussion**

Amplification of the HER-2 proto-oncogene or overexpression of the HER-2/neu protein, which generally correlate with one another, has been identified in 10% to 34% of breast cancers as well as in gastrointestinal, pulmonary, and genitourinary tumors [12–15]. Investigational studies indicate that overexpressed HER-2/neu plays a direct role in the pathogenesis of breast and various other carcinomas.
and aggressiveness of tumors through several lines of experimental evidence: transfection of HER-2/neu into nonneoplastic cells effects malignant transformation [25]; transgenic mice expressing HER-2/neu develop mammary tumors [26]; and the presence of HER-2/neu overexpression may be associated with the development of metastatic disease [26]. FISH and immunohistochemistry are two methods which have been used widely to evaluate HER-2/neu status in clinical samples.

Figure 2. The chromogenic in situ hybridization results of HER-2. (A) and (B). The majority of tumor cells show large clusters of HER-2 gene per nucleus indicating HER-2 gene amplification (×400). (C) Signals of six to 10 individual dots or small clusters of HER-2 gene present per nucleus in >50% of tumor cells indicating HER-2 gene amplification (×400). (D) The tumor cells exhibit no amplification of HER-2 gene (×400).

Figure 3. Prognostic factors of survival for total patients with extrahepatic cholangiocarcinoma by univariate analysis.
laboratories. Both methods have been verified to be sensitive and specific in the detection of either HER-2 amplification or HER-2/neu overexpression using formalin-fixed, paraffin-embedded tissue. Both methods appear to correlate equally well with clinical outcomes in breast cancer patients. Compared with FISH, immunohistochemistry is less time consuming, less expensive, requires minimal instrumentation, and is much easier to perform. However, immunohistochemistry methods can potentially be affected by a host of variables, including tissue fixation, processing, choice of primary antibodies, detection systems, and methods of antigen retrieval [27]. Furthermore, as the suggested scoring system for immunohistochemistry is subjective, its interpretation may vary among observers.

FISH is currently regarded as the ‘gold standard’ for the detection of HER-2 amplification: it is associated with both high sensitivity (96.5%) and specificity (100%) with regard to the detection of HER-2 amplification [28]. However, it also requires a fluorescence microscope and special training in order to interpret the results. It also may be quite difficult to visualize the morphological features of the tumor cells and to separate in situ from invasive carcinoma when evaluating the amplification products via fluorescence. In addition, fluorescence fades quickly and, therefore, does not create a permanent record.

CISH allows detection of gene amplification, chromosome translocations, and chromosome number using conventional peroxidase reactions under the brightfield microscope on formalin-fixed, paraffin-embedded tissue. As the DAB signal is permanent, results may be stored for a long time. The important advantage of CISH is that the analysis of results is fast and easy, and detection of genetic aberration as well as verification of histopathology can be done simultaneously.

Previous reports for the HER-2/neu status in CC were mostly for intrahepatic CC. Ukita et al. [21] demonstrated that HER-2 gene amplification and resultant HER-2/neu protein overexpression compared with noncancerous bile duct were noticed in all of human intrahepatic CC cases they enrolled, however, they could not find any correlation between HER-2/neu expression and clinicopathologic features. Terada et al. [20] also noticed that aberrant expression of HER-2/neu protein was found in 70% of human intrahepatic CC and also showed no significant correlation between the HER-2/neu overexpression and clinicopathologic features. The reports of the HER-2/neu status for extrahepatic CC were sparse.

In our study, the status of HER-2 gene amplification and HER-2/neu protein overexpression for 55 cases of extrahepatic CC including hilar CC were investigated. HER-2/neu protein overexpression was found in 29.1% and HER-2 gene amplification in 70% of human intrahepatic CC and also showed no significant correlation between the HER-2/neu overexpression and clinicopathologic features. The expression rate of HER-2/neu protein seems to be lower in cases with extrahepatic CC.

Previous reports [20, 21] failed to show any correlation between HER-2/neu protein overexpression and clinicopathologic features. Also in our study, there was no significant correlation between the overexpression of HER-2/neu protein and various clinicopathologic features; however, there was a tendency of distant metastases in HER-2/neu protein overexpression group (P = 0.06). As in previous study [21], our results of IHC and CISH were well correlated (Spearman’s rho = 0.718, P < 0.01).
prognostic factors for survival by univariate analyses (Figure 3). However, HER-2 gene amplification by CISH or HER-2/neu overexpression by IHC staining was not significant prognostic factors. But in subgroup analyses, for patients with lymph node metastases, HER-2/neu protein overexpression by immunohistochemistry and HER-2 gene amplification by CISH were poor prognostic factors for survival in univariate and multivariate analysis (Figure 4, Table 3). Previous report [29] indicated that the prognostic impact of HER-2/neu is related only to the first 3–4 years after surgery, as indicated by the peak of early recurrence. The reasons for early recurrences in HER-2/neu-positive tumors have been indicated to rest in events occurring at the time of surgery. Indeed, growth factors released during wound healing have been shown to preferentially stimulate the growth of HER-2/neu-positive tumors [30]. These growth factors are more likely to have a stimulatory effect in patients with disseminated micrometastases (node-positive patients) of an HER-2/neu-positive tumor, which might also explain the prognostic impact of HER-2/neu according to nodal status.

In summary, our study revealed that aberrant expression of HER-2/neu protein and amplification of HER-2 gene were found in considerable proportion of patients with extrahepatic CC. These results support the concept that HER-2/neu protein may participate in extrahepatic cholangiocarcinogenesis. Furthermore, HER-2/neu protein overexpression by HER-2 gene amplification may be independent prognostic factors for survival in subgroup of patients with lymph node metastases. These results might support the need of the prospective randomized controlled trials for the therapeutic effect of humanized monoclonal antibodies of HER-2/neu in a subset of patients with lymph node-positive extrahepatic CC. Further studies on the role of this proto-oncogene in CC will demonstrate whether it can also be used as a target for therapy.

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References