Immunohistochemical Expression of HER-1 and HER-2 in Extrahepatic Biliary Carcinoma

YASUMASA OGO1, YOSHINORI NIO1, SEIJI YANO1, TOMOKO TOGA1, MAKOTO KOIKE1, KOJI HASHIMOTO1, MASAYUKI ITAKURA1 and RIRUKE MARUYAMA2

1Department of Cardiovascular and Digestive Surgery and 2Laboratory of Clinical Pathology, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan

Abstract. The clinicopathological significance of HER-1- and HER-2-overexpressions (OE) (HercepTest score 2+ or 3+) in biliary cancer and their relationship to the efficacy of adjuvant chemotherapy (ACT) were assessed. In 72 biliary cancer (28 gallbladder and 44 bile duct cancer), HER-1 and HER-2 were stained immunohistochemically in formalin-fixed, paraffin-embedded specimens. The ACT included uracil and tegafur (UFT)-based chemotherapies. Out of the 72 cancer, OE was observed in 31 specimens (43%) for HER-1 and 47 (65%) for HER-2. However, their OEs were not correlated with each other. HER-2-OE was inversely correlated with the clinical stage (p=0.0482). HER-1-OE was correlated with distant metastasis (p=0.0263), but not with the clinical stage. Neither the OE of HER-1 or HER-2, nor their co-expression, showed any significant effect in term of patient survival. In the HER-1-OE (–) patients, the survival rate of the ACT group was significantly higher than that of the surgery-alone (SA) group (p=0.0423), but in the HER-1-OE (+) patients, there was no statistical difference in survival rate between the ACT and the SA group. In contrast, HER-2-OE had no significant effect on the efficacy of ACT. Multivariate analysis also demonstrated that the histological grade and ACT were significant variables, but T, N, M and HER-1 and HER-2 were not significant variables. In conclusion, neither HER-1-OE or HER-2-OE were prognostic factors of the biliary cancer. However, HER-1-OE may be a useful marker for the indication of ACT.

Biliary cancer is relatively rare, comprising only 2% of all gastrointestinal malignancies, with incidences in the United States of 2.5 and 2.0 per 100,000 for gallbladder and bile duct cancer, respectively (1, 2). In Japan, the incidences of these malignancies were 2.1% and 0.7% of autopsy cases, respectively (3). Since the majority of patients with biliary cancer often present with advanced disease beyond the curative stage at the time of diagnosis, the prognosis of these patients is extremely poor; the overall 5-year survival rates were only 6% – 12% (1, 4, 5). Despite recent progress in cancer therapy, complete surgical resection remains the best option for long-term survival (2, 6). However, the 5-year survival rate after surgical resection ranged from 20% to 40%, and survival was influenced by the stage of the disease and the histological type (1, 6-10). Radiotherapy and chemotherapy are not effective for the treatment of biliary carcinoma, and there are no standard regimens for adjuvant therapy (2, 10, 11).

Recent progress in biomolecular technology has generated significant evidence implicating tyrosine kinase (TK) as one of the key enzymes, because TK regulates malignant progression in various neoplasms (12). Furthermore, several oncogenes, which encode for growth factor receptors, have TK activity (12). The epidermal growth factor receptor (EGFR, HER-1) and c-erbB-2 (HER-2) are members of the EGFR family with TK activity, and the HER-1 and HER-2 proto-oncogenes encode for 170 and 185 kDa transmembrane glycoproteins, respectively. The overexpression (OE) of these oncoproteins is often a result of gene amplification, and is widely observed in various solid malignancies. HER-1 has been reported to be overexpressed in 37% of breast cancer, 25 - 100% of colorectal cancer, 33 - 81% of gastric cancer, 30 - 50% of pancreatic cancer, 86 - 92% of glioma, 40% - 81% of non-small cell lung cancer (NSCLCs) and 35 - 70% of ovarian cancer (13). In contrast, HER-2 has been reported to...
be overexpressed in 17 - 37% of breast cancers, 12 - 55% of gastric cancers, 27 - 56% of NSCLCs and 32% of ovarian cancers (13, 14). However, to our knowledge, there are only a few studies reporting the expression of HER-2 in biliary cancer (15-17), and no publications have reported the expression of HER-1 in biliary cancer. Therefore, the clinical significance of the OE of these onco-proteins in biliary cancer is still unclear.

The present study was designed to evaluate the relationship between HER-1-OE or HER-2-OE and the clinicopathological features of extrahepatic biliary cancers. Furthermore, the utility of HER-1 and HER-2 as prognostic markers, and their usefulness in determining the potential efficacy of post-surgical adjuvant chemotherapy (ACT) were also examined.

**Materials and Methods**

**Patients.** The patient cohort comprised 72 patients with biliary cancer (28 gallbladder cancer and 44 bile duct cancer): 43 males and 29 females, with a mean age of 67.3 years, ranging between 38 and 81. All patients underwent surgical resection in our department between 1983 and 2003, including 24 cholecystectomies, 24 pancreaticoduodenectomies, 2 hepato-pancreatico-duodenectomies, 8 hepatectomies and 14 total choledochectomies. The patient profiles are summarized in Table I. None of the patients received any cancer therapy prior to surgery. Of the 72 patients, 55 patients (76%) received ACT. The ACT consisted in 40 cases of oral UFT (a mixture of uracil plus tegafur in a proportion of 4:1) alone, 12 UFT plus cyclophosphamide (CPA) and 3 other regimens, which have been approved for chemotherapy against biliary cancers by the Japanese Ministry of Welfare, Health and Labor. The administration of ACT was not randomized and ACT was administered according to the doctor’s preference with informed consent. The pathological diagnosis and stage were classified according to the UICC (pTNM) classification (18). All patients were followed-up in our department and the survival of the patients was surveyed on June 30, 2004. Post-surgical survival was defined as the time that had elapsed from surgery to a biliary cancer-related death.

**Immunohistochemical study.** For the primary antibody (Ab), a rabbit anti-human c-erbB-2 oncoprotein polyclonal antibody (pAb)
(DAKO HercepTest, A0485) and a mouse anti-human EGFR monoclonal Ab (mAb) (DAKO, clone H11, M3563) were purchased from DAKO Corp.

HER-1 was immunostained by an enhanced polymer (DAKO EnVision™ System) procedure, and HER-2 was immunostained using a DAKO HercepTest™ kit, which also uses an enhanced polymer procedure, according to the manufacturer’s guidelines. All of the specimens were fixed in formalin and embedded in paraffin. Four-micron (4 µm)-thick sections were then prepared on glass slides. The specimens were deparaffinized in xylene for 5 min 3 times, and then rehydrated in 100%, 95% and 45% ethanol and finally in phosphate-buffered saline (PBS).

For HER-1 immunostaining, the sections were then treated with proteinase K (DakoproK at 1:5 dilution) at 37°C for 30 min for antigen retrieval. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 5 min. The specimens were then treated with an anti-EGFR mAb at a 1:300 dilution, at 4°C overnight. After rinsing twice with a buffer solution (0.05 M Tris buffer, pH 7.6) for 5 min, the specimens were incubated with a peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins (K4000, DakoCytomation) for 30 min at room temperature, and then rinsed for 5 min and placed in the buffer solution.

For the immunostaining of HER-2, the sections were subjected to heat-induced epitope retrieval by immersing the slides in Dako Epitope Retrieval Solution (0.01 mol/L citrate buffer; pH = 6.0) preheated to 95°C, and then heating in a water bath at 95°C for a total of 40 min, followed by a 20-min cool-down at room temperature. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 5 min. The specimens were then treated with a rabbit anti-c-erbB-2 pAb at a 1:100 dilution for 30 min at room temperature. After rinsing twice with the buffer solution for 5 min, the specimens were incubated with a polymer reagent (HercepTest, a peroxidase-labelled polymer conjugated with goat anti-rabbit immunoglobulins) for 30 min at room temperature, and then rinsed for 5 min and placed in the buffer solution.

The reaction products were visualized by immersing the specimens in 3,3-diaminobenzidine tetrahydrochloride solution for 10 min. After rinsing with distilled water, the specimens were finally counterstained with hematoxylin, and then mounted in Entellan-new (Merck & Co. Inc., Rathway, NJ, USA) with a coverslip.

Evaluation of immunostaining. Positive controls were included in each immunostaining run. For HER-1 immunostaining, freshly resected lung cancer cases, which are known to express HER-1, were used as positive controls. For HER-2 immunostaining, breast cancer cases, which are known to express HER-2, were used. The negative controls were prepared by substituting normal rabbit serum (Dako Negative Control Reagent) and normal mouse serum (Dako Negative Control Reagent) for the primary Abs against HER-2 and HER-1, respectively. Both HER-2 and HER-1 immunostaining were assessed according to the HercepTest™ kit scoring guideline, because both are transmembrane glycoproteins. Only the membrane immunostaining intensity and the pattern were evaluated using the 0 to 3+ scales as illustrated in the guideline. As defined in the guideline, scores of 0 or 1+ were considered negative or weakly-positive, 2+ was moderately-positive, and 3+ was strongly-positive. In the present study, a modification of this scoring system was used such that, to qualify for 2+ and 3+ scoring, complete membrane immunostaining of more than 80% of the tumor cells had to be observed. A score 2+ or 3+ was defined as OE (+).

Statistical analysis. A Chi-square test was used to compare the backgrounds of the patients. Paired and unpaired t-tests and Pearson’s correlation coefficient were used to determine statistical
The cumulative survival rates were calculated by the Kaplan-Meier method. To compare the survival curves, a Cox-Mantel test was used. Multivariate analysis of the maximum likelihood estimates using Cox’s proportional hazard model was used to obtain the conditional risk of biliary carcinoma-related death. All analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA) and a p-value less than 0.05 was considered to be statistically significant.

Results

Representative immunostaining for HER-1 and HER-2 is shown in Figure 1. HER-1 and HER-2 were overexpressed (OE, score 2+ or 3+) in 31 (43%) and 47 (65%) out of 72 patients, respectively. The HER-1-OE and HER-2-OE were not correlated with each other (Table II). The relationship between HER-1-OE or HER-2-OE and various clinico-pathological variables in biliary cancer is summarized in Table I. HER-2-OE was inversely correlated with the clinical stage (p=0.0482). HER-1-OE was correlated with distant metastasis (p=0.0422), but was not correlated with the clinical stage.

The effects of HER-1 and HER-2 expressions on the survival rate and the efficacy of ACT are summarized in Table III. Neither the OE of HER-1 or HER-2, nor their co-expression, showed any significant effect on patient survival (Figure 2).

With regard to the administration of ACT in all cases, there was no significant difference in survival between the ACT group and the surgery-alone (SA) group (p=0.2514) (Figure 3). However, HER-1-OE had a statistically significant effect on the efficacy of ACT and, in the HER-1-OE (−) patients, the survival rate of the ACT group was significantly higher than that of the SA group (p=0.0423). However, in the HER-1-OE(+) patients, there was no difference in survival rate between the ACT and the SA groups. In contrast, HER-2-OE showed no statistically significant effect on the efficacy of ACT (Figure 4).

Multivariate analysis also demonstrated that the histological grade was the only significant variable and that ACT was a variable with a borderline significance, whereas the stage, HER-1 and HER-2 expressions were not significant variables (Table IV).

Discussion

In the present study, HER-1-OE was observed in 31 (43.1%) out of 72 cases of biliary cancer specimens. To our knowledge, no other publications have reported the expression of HER-1 in biliary carcinomas. As mentioned previously, HER-1 has been reported to be overexpressed in several cancers (13), for which HER-1-OE is correlated with advanced disease and a poor prognosis. However, the impact of HER-1 expression on the prognosis is still controversial. In breast cancer, HER-1-OE was associated with a poor prognosis (19); in colorectal cancer, HER-1-OE was significantly associated with the tumor stage, but not with overall survival (20); in ovarian cancer, HER-1 expression had no correlation with clinical parameters and no prognostic impact on survival (21); in esophageal squamous cell carcinoma, HER-1-OE was associated with a well-differentiated histology, but survival was not associated with the HER-1 status (22). In the present study, HER-1-OE was significantly correlated with distant metastasis and also tended to be correlated with nodal involvement and an advanced clinical stage (Table I).
On the other hand, HER-2-OE was observed in 47 (65.3%) out of 72 cases of biliary cancer specimens. However, there are only a few studies reporting the expression of HER-2 in biliary cancer, and these previous reports indicated that the frequency of HER-2-OE in biliary cancer varied from 32.6% to 70% (15-17). These differences in the frequency may be due to differences in the experimental methods, the use of different Abs for immunostaining and the different criteria used for evaluation. To our knowledge, the present study is the first to use the HercepTest™ for the evaluation of biliary cancers. In the present study, there were no significant correlations between HER-2-OE and clinicopathological factors, such as clinical stage and histological grade, in agreement with the results from previous reports (15-17).

HER-2 was also reported to be overexpressed in certain cancers (13, 14). Many reports have demonstrated that HER-2-OE was associated with a shortened survival in cancer of the breast (23-25), whereas the prognostic significance of HER-2-OE in other malignancies, such as gastric (26-31), pancreatic (32-34) and ovarian cancer (35-37), has not been clearly established due to conflicting results. In biliary cancer, HER-2-OE was reported to be significantly associated with a shortened survival rate in only one study (15) and the same study failed to find any prognostic significance for HER-2-OE in a multivariate analysis. In the present study, there were no significant
differences in survival rates between the HER-2-OE (+) cases and the (-) cases. Moreover, multiparametric survival analysis also showed that HER-2-OE was not a significant variable. Considering these results, we conclude that the c-erbB-2 oncogene may not play a very important role in the progression and maintenance of biliary cancer.

Several reports have indicated that the HER-1 or HER-2 expression status was significantly implicated in the efficacy of various anticancer therapies. Previous trials have demonstrated that ACT was not effective for the treatment of biliary cancer (10, 38, 39), a finding compatible with our results. However, subgroup analysis demonstrated that in the HER-1-OE (-) group, ACT was correlated with a significantly better outcome, and this report is the first to show that HER-1-OE may be a useful marker indicating the benefit of ACT in biliary cancer. However, HER-2-OE had no significant effect on the efficacy of ACT.

A previous study reported that HER-1-OE was correlated with resistance to chemotherapy in ovarian cancer in vitro (40). It has been reported that HER-1 expression was a strong biomarker for a poor prognosis and resistance to chemotherapy and radiotherapy in head and neck cancer and glioma (41-43). Furthermore, it has been reported that HER-1 expression was correlated with the resistance of ovarian cancer cell lines to mitomycin-C in vitro (44). These results are compatible with the present results. In the present study, the patients received UFT-based chemotherapies. UFT is a mixture of uracil plus tegafur. Tegafur is a prodrug of 5-fluorouracil (5-FU), and is metabolized into 5-FU in the body. To our knowledge, it is unclear whether HER-1-OE is associated with chemosensitivity of the tumor cells to 5-FU. Since the delivery of ACT was not randomized in this study, there is the possibility that a bias in patient selection may have caused the difference in prognosis with or without ACT. Accordingly, to confirm the present results, any future study on the delivery of ACT should be randomized.

Table IV. Multivariate analysis of all patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Risk rate (95% confidence)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>1.238 (0.781-1.964)</td>
<td>0.3635</td>
</tr>
<tr>
<td>Histological grade</td>
<td>1.553 (1.055-2.288)</td>
<td>0.0257</td>
</tr>
<tr>
<td>HER-1 score</td>
<td>1.160 (0.888-1.517)</td>
<td>0.2767</td>
</tr>
<tr>
<td>HER-2 score</td>
<td>1.072 (0.758-1.515)</td>
<td>0.6938</td>
</tr>
<tr>
<td>Adjuvant chemotherapy</td>
<td>0.451 (0.195-1.043)</td>
<td>0.0625</td>
</tr>
<tr>
<td>Age</td>
<td>0.988 (0.951-1.026)</td>
<td>0.5165</td>
</tr>
<tr>
<td>Gender</td>
<td>1.559 (0.727-3.341)</td>
<td>0.2537</td>
</tr>
</tbody>
</table>

Figure 4. Effects of HER-1 and HER-2 overexpressions on the efficacy of adjuvant chemotherapy (ACT).
HER-1: HER-1-OE(-)/ACT(+), n=26; HER-1-OE(-)/ACT(-), n=15; HER-1-OE(+)/ACT(+), n=29; HER-1-OE(+)/ACT(-), n=2.
HER-2: HER-2-OE(-)/ACT(+), n=18; HER-2-OE(-)/ACT(-), n=7; HER-2-OE(+)/ACT(+), n=37; HER-2-OE(+)/ACT(-), n=10.
In the HER-1-OE(-) patients, the survival rate of the ACT group was significantly higher than that of the surgery-alone (SA) group (p=0.0423).
With regard to the effects of HER-2-OE on various anticancer therapies, HER-2-OE (+) breast cancers benefit from anthracyclin treatment and tend to be resistant to endocrine therapy (45). Furthermore, it has also been reported that patients with HER-2 amplification and HER-1-OE were slightly less responsive to endocrine therapy with tamoxifen (TAM) (46), and it has also been reported that TAM behaved as an estrogen agonist in HER-2-OE (+) breast cancer, resulting in de novo resistance (47). It was also reported that HER-2-OE was correlated with the resistance of ovarian cancer cell lines to cisplatin in vitro (44). However, it has also been reported that radiosensitivity was increased in HER-2-OE (+) breast cancer cells as measured by in vitro colony-forming assays (48).

Recently, a variety of targeting therapies against HER-1 and HER-2 has been used for the treatment of these malignancies with HER-1-OE or HER-2-OE. Accordingly, the present results suggest that biliary cancer may also be indicated for targeting therapies.

In conclusion, neither HER-1-OE nor HER-2-OE is a prognostic factor for biliary cancer. However, HER-1-OE may be a useful marker to indicate the usefulness of ACT.

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References


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