Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma

Epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and human epidermal growth factor receptor 2 (HER2) have been considered as potential therapeutic targets in cholangiocarcinoma, but no studies have yet clarified the clinicopathological or prognostic significance of these molecules. Immunohistochemical expression of these molecules was assessed retrospectively in 236 cases of cholangiocarcinoma, as well as associations between the expression of these molecules and clinicopathological factors or clinical outcome. The proportions of positive cases for EGFR, VEGF, and HER2 overexpression were 27.4, 53.8, and 0.9% in intrahepatic cholangiocarcinoma (IHCC), and 19.2, 59.2, and 8.5% in extrahepatic cholangiocarcinoma (EHCC), respectively. Clinicopathologically, EGFR overexpression was associated with macroscopic type (P = 0.0120), lymph node metastasis (P = 0.0006), tumour stage (P = 0.0424), lymphatic vessel invasion (P = 0.0371), and perineural invasion (P = 0.0459) in EHCC, and VEGF overexpression with intrahepatic metastasis (P = 0.0224) in IHCC. Multivariate analysis showed that EGFR expression was a significant prognostic factor (hazard ratio (HR), 2.67; 95% confidence interval (CI), 1.52–4.69; P = 0.0006) and also a risk factor for tumour recurrence (HR, 1.89; 95% CI, 1.05–3.39; P = 0.0335) in IHCC. These results suggest that EGFR expression is associated with tumour progression and VEGF expression may be involved in haematogenic metastasis in cholangiocarcinoma.

Keywords: cholangiocarcinoma; epidermal growth factor receptor; vascular endothelial growth factor; human epidermal growth factor receptor 2; immunohistochemistry; prognosis

Cholangiocarcinoma arises from the ductal epithelium of the bile duct tree and is classified anatomically into intrahepatic cholangiocarcinoma (IHCC) and extrahepatic cholangiocarcinoma (EHCC). The incidence and mortality rates of cholangiocarcinoma, especially those of IHCC, are increasing worldwide (Khan et al., 2003; Gwak et al., 2001; Aishima et al., 2002; Nakazawa et al., 2003; Tang et al., 2005). Complete resection is the only way to cure the disease at present. Moreover, because cholangiocarcinoma is difficult to diagnose at an early stage and extends diffusely, most patients have unresectable disease at clinical presentation, and prognosis is very poor (5-year survival is 0–40% even in resected cases) (Khan et al., 2005; Sirica, 2005). Therefore, novel effective therapeutic strategies are urgently required to improve the prognosis. Among potential therapeutic targets, several studies have revealed overexpression of epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) protein, amplification, and mutation of these genes (Ito et al., 2001; Aishima et al., 2002; Ukita et al., 2002; Altimari et al., 2003; Gwak et al., 2005; Nakazawa et al., 2005; Leone et al., 2006) as well as overexpression of vascular endothelial growth factor (VEGF) protein (Hida et al., 1999; Tang et al., 2006) in cholangiocarcinoma.

Epidermal growth factor receptor and HER2 are members of the ErbB receptor tyrosine kinase family. Binding of ligands, such as epidermal growth factor and transforming growth factor alpha (TGFα), to their extracellular ligand-binding domain initiates intracellular signalling cascades, leading to progression, proliferation, migration, and survival of cancer cells (Olayioye et al., 2000; Yarden and Sliwkowski, 2001). Vascular endothelial growth factor plays a key role in tumor-associated neo-angiogenesis, which contributes to providing a tumor with oxygen, nutrition, and a route for metastasis. It binds to VEGFR (vascular endothelial growth factor receptor), and leads to survival, proliferation, and migration of endothelial cell (Tabernero, 2007). Expression of these molecules has been reported to have prognostic significance in several cancers (Gusterson et al., 1992; Han et al., 2001; Nicholson et al., 2001; Des Guetz et al., 2006; Mohammed et al., 2007). Recently, agents targeted at these molecules have been used clinically, such as trastuzumab in breast cancer (Gonzalez Angulo et al., 2006), gefitinib, and erlotinib in non-small cell lung cancer, and bevacizumab in colorectal cancer (Tabernero, 2007). In cholangiocarcinoma, a phase II study of erlotinib (Philip et al., 2006) and some case reports of combined chemotherapy including cetuximab (Sprinzl et al., 2006; Huang et al., 2007) have been reported.

However, no previous studies have clarified associations between the expression of these molecules and clinicopathological
factors or prognosis in patients with cholangiocarcinoma. To elucidate the biological significance and potential of these molecules as therapeutic targets, we investigated EGFR/VEGF/HER2 expression and attempted to elucidate their associations with various clinical features as well as patient survival in 236 cases of cholangiocarcinomas.

MATERIALS AND METHODS

Patients

A total of 236 patients with cholangiocarcinoma (male 160; female 76) who had undergone tumour resection and been diagnosed histologically as having adenocarcinoma of the bile duct at the National Cancer Center Hospital, Tokyo, between January 1991 and August 2004, were enrolled in the present study. Median patient age and follow-up period were 65 years and 875 days, and median tumour sizes of IHCC and EHCC were 4.8 and 3.0 cm, respectively. Detailed characteristics of patient with IHCC and EHCC are presented in Tables 1 and 2. All patients were followed for more than 100 days. Follow-up examination was performed using computed tomography, abdominal ultrasonography, and measurement of the serum carcinoembryonic antigen and carbohydrate antigen 19–9 (CA19-9) levels every 3–6 months. Recurrence was diagnosed by clinical, radiological, or pathological methods, but mainly by radiological evaluation including computed tomography and ultrasonography. Clinical and pathological profiles were obtained from the database of hepatobiliary tumours based on the medical records of the patients. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan, and written informed consent was obtained from all patients.

All cases were anatomically classified into two groups: IHCC and EHCC. Tumours arising from the bilateral hepatic duct or distal common bile duct were classified as EHCC. The numbers of IHCC and EHCC cases were 106 and 130, respectively.

Histological assessment

Tumour staging and histological classification were assessed according to TNM Classification of Malignant Tumours (Sobin and Wittekind, 2002) defined by the International Union Against Cancer (UICC) and the World Health Organization Histological Classification of Tumours (Hamilton and Altonen, 2000). Macroscopic types of IHCC were defined with reference to General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan, 2003): (1) the mass-forming type (MF), which develops an apparent tumour in the liver; (2) the periductal infiltrating type (PI), which spreads along the bile duct; (3) the intraductal growth type (IG), which is confined within the bile duct, and divided into two groups: the mass-forming group (MF and MF mixed with PI or IG) and the non-mass forming group (PI and/or IG). Macroscopic types of EHCC were divided into polyoid type and non-polyoid type (including nodular, scirrhus constricting, and infiltrating types). Other clinicopathological factors were categorised into groups that are presented in Table 1 (IHCC) and Table 2 (EHCC). Because the classifications and clinicopathological factors used in IHCC and EHCC are different, statistical analyses were performed separately.

Immunohistochemistry

Immunohistochemistry (IHC) for EGFR, VEGF, and HER2 was performed using a polymer-based method (Envision™ + Dual Link System-HRP (Dako, DK-2600 Glostrup, Denmark)). Sources and dilutions of primary antibodies were as follows: anti-EGFR (mouse monoclonal, clone 31G7; Zymed, South San Francisco, CA, USA; 1:100), anti-VEGF (rabbit polyclonal; Zymed; 1:50), and anti-HER2 (rabbit polyclonal; Dako; 1:300). Formalin-fixed, paraffin-embedded serial tissue sections (4 µm) were placed on silane-coated slides for IHC. Sections cut through the maximum tumour diameter were selected for IHC evaluation. The sections were deparaffinised and rehydrated in xylene and grade-diluted ethanol (50–100%), and submerged for 20 min in 0.3% hydrogen peroxide with absolute methanol to block endogenous peroxidase activity. Antigen retrieval for EGFR, VEGF, and HER2 was carried out by adding Digest-all™3 pepsin solution (Zymed) at 37°C for 10 min for EGFR, near boiling in 0.01 M citrate buffer (pH 6.0) for 15 min for VEGF, and heating in 0.01 M citrate buffer at 121°C for 10 min by pressure cooker for HER2. After protein blocking, the sections were incubated with each primary antibody at room temperature for 1 h, followed by incubation with

| Factors | Categories | Population |
|---------|------------|------------|
| Age     | <65 years old | 54 (50.9%) |
|         | ≥65 years old | 52 (49.1%) |
| Gender  | Male        | 64 (60.4%) |
|         | Female      | 42 (39.6%) |
| Tumour size | ≤5.0 cm    | 55 (55.6%) |
|         | >5.0 cm     | 44 (44.4%) |
| Macroscopic type | Non-mass forming | 17 (16.0%) |
|         | Mass forming | 89 (84.0%) |
| Invasion of portal vein | Negative | 23 (21.9%) |
|         | Positive    | 82 (78.1%) |
| Invasion of hepatic vein | Negative | 56 (54.9%) |
|         | Positive    | 46 (45.1%) |
| Intrahepatic metastasis | Negative | 75 (70.8%) |
|         | Positive    | 31 (29.2%) |
| Lymph node metastasis | Negative | 62 (58.5%) |
|         | Positive    | 44 (41.5%) |
| UICC pT | 1 + 2      | 71 (68.3%) |
|         | 3+4        | 33 (31.7%) |
| UICC stage | 1 + 2      | 45 (42.5%) |
|         | 3A+3B+3C   | 61 (57.5%) |
| Histological classification | Well | 22 (20.8%) |
|         | Mod        | 79 (74.5%) |
|         | Por        | 5 (4.7%) |
| Lymphatic vessel invasion | Negative | 20 (18.9%) |
|         | Positive   | 86 (81.1%) |
| Venous invasion | Negative | 19 (17.9%) |
|         | Positive   | 87 (82.1%) |
| Perineural invasion | Negative | 29 (27.4%) |
|         | Positive   | 77 (72.6%) |
| Hepatic surgical margin | Negative | 89 (84.0%) |
|         | Positive   | 17 (16.0%) |
| Bile duct margin | Negative | 91 (85.8%) |
|         | Positive   | 15 (14.2%) |
Evaluation of immunohistochemistry

All sections were evaluated by DY, HO, and TS without the knowledge of any clinical or pathological information, and cases for which consensus could not be reached were discussed to decide the evaluation. Based on the Herceptest™ (Dako) criteria, intensities of both EGFR and HER2 were defined as follows: 0, no membrane staining or membrane staining in <10% cancer cells; 1+, faint and partial membrane staining in >10% cancer cells; 2+, moderate and complete membrane staining in >10% cancer cells; 3+, strong and complete membrane staining in >10% cancer cells. Intensities of VEGF were defined as follows: 0, no cytoplasmic staining or cytoplasmic staining in ≤30% cancer cells; 1+, faint cytoplasmatic staining, equivalent to the intensity of normal bile duct epithelium within the same section, in >30% cancer cells; 2+, moderate cytoplasmatic staining in >30% cancer cells; 3+, strong cytoplasmatic staining in >30% cancer cells. For cases showing mixed intensity, the predominant intensity was selected as the final IHC score. A final IHC score of 2+ or 3+ was defined as positive for expression of each protein.

Statistical analysis

Associations between results of IHC and clinical-pathological factors were assessed by χ² test. Cumulative survival rates and survival curves were calculated by the Kaplan–Meier method, and log-rank test was performed for the comparison of survival curves. Cox’s proportional hazard model was performed to estimate hazard ratio (HR) and 95% confidence interval (CI) of each outcome (death and recurrence). Multivariate analyses were performed using the factors identified to be risk factors for each outcome by univariate analyses, without UICC pT and UICC Stage, which are composed of other factors. All P-values reported are two-sided, and significance level was set at P < 0.05. All statistical analyses were performed with the Statview 5.0 statistical software package (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Expression of EGFR, VEGF, and HER2 protein in cholangiocarcinoma

Representative cases of positive staining for each protein are shown in Figure 1 (A, EGFR; B, HER2; C, VEGF). Epidermal growth factor receptor, VEGF, and HER2 were expressed in 29 (27.4), 57 (53.8), and 1 (0.9%) of the 106 IHCCs, respectively, and in 25 (19.2), 77 (59.2), and 11 (8.5%) of the 130 EHCCs, respectively. Microscopically, EGFR was mostly overexpressed in the moderately and/or poorly differentiated component, which is characterised by infiltration (52 of 54 EGFR-positive cases, Figure 1D), whereas only two cases showed EGFR overexpression in the well-differentiated component. In contrast, HER2 was preferentially expressed in the well-differentiated component. In 6 of 12 HER2-positive cases, HER2 was expressed only in well-differentiated component (Figure 1E), and 5 progressive cases showed positive HER2 staining in both the well and moderately and/or poorly differentiated components and 1 case only in moderately differentiated component. There was no association between VEGF expression and histological features.

Associations between EGFR, VEGF, and HER2 expression and clinicopathological factors

Statistical analyses of HER2 were performed only in EHCC cases because of the small number of HER2-positive cases in IHCC. In IHCC, VEGF expression was significantly associated with intrahepatic metastasis (P = 0.0224). There was no significant association between EGFR expression and any clinicopathological factors. In EHCC, EGFR expression was significantly associated with macroscopic type (0% in the polypoid type, 24.0% in the non-polypoid type; P = 0.0120), lymph node metastasis (P = 0.0006), UICC Stage (P = 0.0424), lymphatic vessels invasion (P = 0.0371), and perineural invasion (P = 0.0459). Human epidermal growth factor receptor 2 expression was significantly associated with...
macroscopic type (23.8% in the polypoid type, 5.8% in the non-polypoid type; \( P = 0.0078 \)), histological classification (25% in papillary adenocarcinoma, 9.7% in well differentiated adenocarcinoma, 3.2% in moderately differentiated adenocarcinoma, 5.9% in poorly differentiated adenocarcinoma; \( P = 0.0237 \)), and invasion to other organs (3.9% in invasive cases, 15.1% in non-invasive cases; \( P = 0.0242 \)). VEGF expression was not significantly associated with any factors in EHCC.

Detailed results of associations between EGFR/VEGF/HER2 expression and clinicopathological factors are shown in Supplementary information 1 (IHCC) and Supplementary information 2 (EHCC).

### Univariate and multivariate analyses regarding overall survival and tumour recurrence in cholangiocarcinoma

The number of dead and the median survival time were 70 cases and 724 days in IHCCs, and 76 cases and 1197 days in EHCCs, respectively. The number of recurrence and the median recurrence time were 64 cases and 522 days in IHCCs, and 78 cases and 960 days in EHCCs, respectively.

Overall 5-year cumulative survival for patients with IHCC and EHCC was 33.0 and 41.6%, respectively, and no significant difference was identified between the groups (\( P = 0.0599 \)). The survival curves stratified by EGFR expression status are shown as Figure 2. Five-year survival for patients with EGFR-positive and EGFR-negative tumours was 17.7 and 47.1% for IHCC, and 26.4 and 45.6% for EHCC, respectively. There was a significant difference between EGFR-positive and -negative cases for both IHCC (\( P = 0.0008 \)) and EHCC (\( P = 0.0204 \)).

The results of multivariate analyses following univariate analyses regarding overall survival and tumour recurrence are shown in Table 3 (IHCC) and Table 4 (EHCC).

In IHCC, 13 factors including EGFR expression were identified as significantly prognostic by univariate analysis. Multivariate analysis revealed that EGFR expression was an independent prognostic factor (HR, 2.67; 95% CI, 1.52 – 4.69; \( P = 0.0006 \)), along with mass-forming macroscopic group (HR, 2.96; 95% CI, 1.06 – 8.31; \( P = 0.0390 \)), intrahepatic metastasis (HR, 2.91; 95% CI, 1.60 – 5.29; \( P = 0.0005 \)), and lymph node metastasis (HR, 1.96; 95% CI, 1.04 – 3.69; \( P = 0.0375 \)). In EHCC, 14 factors including EGFR expression were identified as significantly prognostic by univariate analysis. Multivariate analysis revealed that lymph node metastasis (HR, 2.03; 95% CI, 1.16 – 3.55; \( P = 0.0133 \)) and a histological classification of moderately differentiated adenocarcinoma (HR for papillary adenocarcinoma, 4.23; 95% CI, 1.08 – 16.50; \( P = 0.0380 \)) and poorly differentiated adenocarcinoma (HR for papillary adenocarcinoma, 13.22; 95% CI, 3.10 – 56.45; \( P = 0.0005 \)) were significant prognostic factors.

Multivariate analysis following univariate analysis for risk factors of tumour recurrence revealed that EGFR expression in IHCC was a significant risk factor of tumour recurrence (HR, 1.89;
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422, 2002; Morimoto

Expression of EGFR or HER2 is known to be a prognostic factor in IHCC (Gusterson et al., 1992; Nicholson et al., 2001), but no previous study has clarified the influence of these molecules on prognosis in cholangiocarcinoma (Ito et al., 2001; Altimari et al., 2003; Nakazawa et al., 2005), probably because cholangiocarcinoma is a relatively rare cancer and collection of a large cohort is difficult. Indeed, most previous studies were performed on the basis of only 30 cases at most. Although it is unclear why EGFR expression in IHCC is an independent prognostic factor, it may be associated with frequent relapse of cancer because EGFR expression is also a risk factor for tumour recurrence.

DISCUSSION

This study, analysing EGFR/VEGF/HER2 expression in the largest cohort of cholangiocarcinoma reported so far, showed for the first time that EGFR expression in IHCC is significantly associated with poor prognosis. In addition, our study confirmed previously reported prognostic factors in cholangiocarcinoma, such as macroscopic type, intrahepatic metastasis, lymph node metastasis, and histological classification (Yamamoto et al., 1998; Ohtsuka et al., 2003; Morimoto et al., 2003; DeOliveira et al., 2007). Expression of EGFR or HER2 is known to be a prognostic factor in some cancers (Gusterson et al., 1992; Nicholson et al., 2001), but no previous study has clarified the influence of these molecules on prognosis in cholangiocarcinoma (Ito et al., 2001; Altimari et al., 2003; Nakazawa et al., 2005), probably because cholangiocarcinoma is a relatively rare cancer and collection of a large cohort is difficult. Indeed, most previous studies were performed on the basis of only 30 cases at most. Although it is unclear why EGFR expression in IHCC is an independent prognostic factor, it may be associated with frequent relapse of cancer because EGFR expression is also a risk factor for tumour recurrence.

In contrast to IHCC, EGFR expression was not an independent prognostic factor in EHCC, but was associated with clinical features that may represent tumour progression and invasion, such as lymph node metastasis and apparent stromal invasion in EHCC. Because cancer tissue tends to be heterogeneous, histological diagnosis is generally decided on the basis of the degree of differentiation that predominates. In order to elucidate the biological significance of each protein, we microscopically examined positive cases in detail and compared their expression with histological components, and found that EGFR tended to be expressed in the poorly differentiated component, which is characterised by infiltration in both IHCC and EHCC. Similar results have been reported in bladder cancer (Neal et al., 1985), oesophageal adenocarcinoma (Wilkinson et al., 2004), and IHCC (Ito et al., 2001), although the studies were based on small cohorts. These findings indicate that EGFR expression may be a relatively late event in the development of cholangiocarcinoma and
previously reported that poor differentiation is associated with invasion and progression. Because it has been reported. In this study, the progression-free rate at 6 months as a primary end point was 17% (7/42) despite the fact that disease condition was severe, and the disease control rate was 50% (20/42) (Philip et al., 2006). This study suggested the clinical applicability of the EGFR inhibitor to cholangiocarcinoma. Several clinical trials demonstrating the efficacy of VEGF inhibition for other cancers have been reported (Hurwitz et al., 2004; Sandler et al., 2006), and VEGF upregulation in tumour cells is considered to be a mechanism of resistance to EGFR inhibitors (Villoria Petit et al., 2001). Therefore, dual inhibition of both EGFR and VEGF may exert a synergistic effect.

In summary, we have shown that EGFR and VEGF expression is relatively common in cholangiocarcinoma. Moreover, in IHCC, EGFR expression is an independent prognostic factor and VEGF expression is associated with intrahepatic metastasis. In HC, EGFR expression is associated with clinical factors involved in tumour progression and invasion. Our results suggest the clinical applicability of the EGFR inhibitor to cholangiocarcinoma. Several clinical trials demonstrating the efficacy of VEGF inhibition for other cancers have been reported (Hurwitz et al., 2004; Sandler et al., 2006), and VEGF upregulation in tumour cells is considered to be a mechanism of resistance to EGFR inhibitors (Villoria Petit et al., 2001). Therefore, dual inhibition of both EGFR and VEGF may exert a synergistic effect.

Table 4. Multivariate analyses regarding overall survival and tumour recurrence in EHCC (Cox’s proportional hazard model).

| Overall survival | Tumour recurrence |
|------------------|-------------------|
| **HR** | **95% CI** | **P-value** | **HR** | **95% CI** | **P-value** |
| Tumour size | | | | | |
| ≤3.0 cm | 1.00 | — | — | — | — |
| >3.0 cm | 1.29 | 0.71–2.35 | 0.41 | — | — |
| Microscopic type | | | | | |
| Polypoid | 1.00 | — | — | — | — |
| Non-polypoid | 0.44 | 0.16–1.26 | 0.13 | — | — |
| Depth of tumour invasion | | | | | |
| Within FM | 1.00 | — | — | — | — |
| Beyond FM | 1.26 | 0.19–8.60 | 0.81 | 1.16 | 0.24–5.57 | 0.85 |
| Invasion of portal vein | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.48 | 0.81–2.69 | 0.20 | 1.59 | 0.92–2.75 | 0.94 |
| Lymph node metastasis | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 2.03 | 1.16–3.55 | 0.0133 | 1.75 | 1.03–2.98 | 0.0394 |
| Histological classification | | | | | |
| Papillary | 1.00 | — | — | — | — |
| Well differentiated | 3.40 | 0.85–13.66 | 0.0849 | 0.91 | 0.33–2.51 | 0.85 |
| Moderately differentiated | 4.23 | 1.08–16.50 | 0.0380 | 1.19 | 0.47–3.02 | 0.72 |
| Poorly differentiated | 13.22 | 3.10–56.45 | 0.0005 | 2.80 | 0.99–7.87 | 0.0516 |
| Lymphatic vessel invasion | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.78 | 0.29–11.10 | 0.54 | 2.36 | 0.45–12.37 | 0.31 |
| Venous invasion | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 3.93 | 0.81–19.12 | 0.0898 | 1.89 | 0.52–6.92 | 0.34 |
| Perineural invasion | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.94 | 0.58–6.53 | 0.29 | 0.98 | 0.38–2.51 | 0.97 |
| Dissected periductal structures margin | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.20 | 0.67–2.17 | 0.54 | 1.81 | 1.03–3.16 | 0.0383 |
| Invasion to other organs | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.02 | 0.53–1.94 | 0.96 | 0.94 | 0.53–1.69 | 0.84 |
| EGFR expression | | | | | |
| Negative | 1.00 | — | — | — | — |
| Positive | 1.04 | 0.55–1.96 | 0.90 | — | — |

HR = hazard ratio; CI = confidence interval; FM = fibromuscular layer.
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