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ANATOMICAL PATHOLOGY

Gene copy number variation and protein overexpression of EGFR and HER2 in distal extrahepatic cholangiocarcinoma

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Summary

EGFR and HER2 are among the most promising therapeutic targets in solid cancers. The expression status of EGFR and HER2 are associated with the prognosis, and with a number of clinicopathological factors, in many cancers. However, few studies have examined this association in distal extrahepatic cholangiocarcinoma (EHCC). Therefore, we investigated EGFR and HER2 protein expression and gene copy number variation (CNV) in distal EHCC. We also studied the association of these factors with clinicopathological parameters and prognosis. Immunostaining, using antibodies against EGFR and HER2, was performed on 84 cases of distal EHCC. All positive (3+) and equivocal (2+) EGFR and HER2 expression cases, together with randomly selected negative (1+ and 0) cases, were evaluated for *EGFR* and *HER2* CNV. Among distal EHCC samples, 6.0% ($n=5$) were positive (3+) for EGFR expression and 6.0% ($n=5$) were equivocal (2+). HER2 expression was positively identified in 2.4% of samples ($n=2$), and was equivocal in 1.2% of samples ($n=1$). All cases of positive EGFR expression showed amplification ($n=1$) or high polysomy ($n=4$) involving the *EGFR* gene; three cases (60%) of equivocal EGFR expression showed high polysomy of the *EGFR* gene. All cases of positive or equivocal HER2 expression ($n=3$, 3.6%) showed amplification of the *HER2* gene. In univariate analysis, EGFR expression and CNV were associated with shorter cancer-specific overall survival ($p=0.003$ and $p=0.018$, respectively). Multivariate analysis also showed that EGFR CNV was a significant prognostic factor in distal EHCC ($p=0.015$). Although further study is warranted, our findings suggest that EGFR expression and CNV are factors associated with poor prognosis, and that anticancer therapeutics against EGFR and HER2 receptors may be promising therapeutic options for patients with distal EHCC.

Key words: EGFR; HER2; cholangiocarcinoma; distal; copy number variation.

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INTRODUCTION

Extrahepatic cholangiocarcinoma (EHCC) is one of the most aggressive solid cancers. It represents an important cause of cancer-related mortality in Asia, but occurs less frequently in the United States and Western Europe. The Republic of Korea has the highest age-standardised incidence rate of EHCC (3.3 per 100,000 in men, 1.5 in women), about 5–7 times greater than that of the USA (0.5 per 100,000 in men, 0.3 in women).[1] In spite of therapeutic advances, the prognosis of EHCC has shown little improvement. Over recent decades, great advances have been made in our understanding of cancer genetics, and the identification of particular oncogenes has led to the development of anticancer therapeutics.

The identification of the epidermal growth factor receptor (EGFR) superfamily is an important example. EGFR/erbB1 and HER2/erbB2, the two most well-studied members of the EGFR superfamily, are very effective therapeutic targets in many solid cancers. Several anticancer drugs directed against EGFR ('EGFR inhibitors') have been developed and used widely, including gefitinib, erlotinib, afatinib, brigatinib, and icotinib for lung cancer,[2,3] and cetuximab for colon cancer.[4] HER2 is the target of the monoclonal antibody trastuzumab (marketed as Herceptin), which is very effective against HER2 positive breast[5] and gastric cancers.[6] Meanwhile, EGFR and HER2 status, including levels of protein expression, presence of specific mutations, and copy number variation (CNV), have been measured and evaluated together with pharmacological responses to EGFR and HER2 inhibitors. Expression and/or activation of EGFR is associated with metastasis, poor prognosis, and resistance to chemotherapy in many cancers.[7] HER2 expression is also correlated with poor outcomes and more aggressive breast and gastric cancers.[8,9] Some recent case reports and phase III trials have reported promising results after targeting HER2[10] or EGFR.[11] However, few studies have investigated the status or biological role of EGFR and HER2 in EHCC.

We investigated the protein expression status and gene copy number of EGFR and HER2 using immunohistochemical (IHC) stains and fluorescence *in situ* hybridisation (FISH) or silver *in situ* hybridisation (SISH) in 84 cases of

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distal bile duct carcinoma. We evaluated whether there was a significant association between EGFR and HER2 status and clinicopathological parameters and outcomes in distal EHCC patients.

MATERIALS AND METHODS

Case selection

We reviewed 84 cases of surgically resected distal EHCC from the Samsung Seoul Medical Center between 2003 and 2006. Chung *et al.*[12] reported that a survival difference was evident between patients receiving a Whipple procedure and those receiving pylorus-preserving pancreatoduodenectomy (PPPD) ($p = 0.006$), despite the fact that tumour staging was not significantly different between groups. Our case records showed that that the use of the Whipple procedure declined from 51 to 18 cases after 2002, while PPPD had increased from 30 to 138 cases. For this reason, and to rule out a cohort effect, we selected cases from the period after 2002.

All patients underwent resections for distal EHCC with curative intent. Only carcinomas with an epicentre in the distal EHCC were included in this study. Distal EHCC was defined as the region of the bile duct distal to the insertion of the cystic duct. Carcinomas with epicenters in the ampulla of Vater, gallbladder, or hilar area were excluded. Patients with either more than one primary cancer or neuroendocrine tumours were also excluded. All cases were independently reviewed by two pathologists (AM and KJ), and the diagnoses were confirmed in all instances. Clinicopathological parameters, including age, gender, pathological T stage (pT), N stage (pN), completeness of resection, and overall survival were assessed by the same pathologists (AM and KJ). No distant metastases were identified at the time of surgery.

Tissue microarray construction

Tissue microarrays were constructed from formalin fixed, paraffin embedded tissue blocks. For each tumour, a representative tumour area was carefully selected from a haematoxylin and eosin (H&E) stained section of a donor block. Each case was represented by two cores, each 3 mm in diameter.

Immunohistochemistry

Immunohistochemistry with a mouse monoclonal antibody against EGFR [Confirm anti-EGFR (3C6) kit; Ventana, USA] and a rabbit monoclonal antibody for HER2/NEU [XT Optiview DAB IHC V4 (4B5) kit; Ventana] was performed on tissue microarray sections, using an automated Benchmark platform (Ventana Medical Systems) according to the manufacturer's recommendations. The grade of EGFR and HER2 staining was defined according to the Trastuzumab for Gastric Cancer (ToGA) trial (0, no membrane staining or membrane staining in $\leq$10% of cancer cells; 1+, faint and partial membrane staining in >10% of cancer cells; 2+, moderate and complete membrane staining in >10% of cancer cells; and 3+, strong and complete membrane staining in >10% of cancer cells).[13] Two surgical pathologists (AM and HK) verified the IHC staining and independently scored the level of protein expression.

FISH for EGFR gene

The ZytoLight SPEC EGFR/CEN 7 Dual Color Probe, designed for detection of *EGFR* gene amplification, was used (Zytovision, Germany). We selected 21 cases, including all positive (3+) and equivocal (2+) EGFR expression cases, and randomly selected negative (1+ and 0) cases for *EGFR* FISH. Sections (4 μm thick) were cut from the paraffin blocks and dewaxed. Consecutive unstained sections were stained according to the manufacturer's protocols. The SpectrumGreen labelled *EGFR* probe locates to 7p11.2 and the SpectrumOrange labelled probe is against the centromeric region of chromosome 7 (CEP7). Patients were classified into six FISH strata with ascending numbers of copies of the *EGFR* gene per cell, using the frequency of tumour cells with specific numbers of copies of the *EGFR* gene and CEP7 centromere: (1) disomy ($\leq$2 copies in >90% of cells); (2) low trisomy ($\leq$2 copies in $\geq$40% of cells, 3 copies in 10–40% of cells, $\geq$4 copies in <10% of cells); (3) high trisomy ($\leq$2 copies in $\geq$40% of cells, 3 copies in $\geq$40% of cells, $\geq$4 copies in <10% of cells); (4) low polysomy ($\geq$4 copies in 10–40% of cells); (5) high polysomy ($\geq$4 copies in $\geq$40% of cells); and (6) gene amplification (defined by presence of tight *EGFR* gene clusters and a ratio of *EGFR* gene to centromere of $\geq$2, or by $\geq$15 copies of *EGFR* per cell in $\geq$10% of analysed cells).[2]

SISH for HER2 gene

An Inform HER2 Dual ISH assay kit (Ventana) was used. Cases were selected, and sections prepared and stained as described for FISH. SISH slides were evaluated with conventional bright field microscopy using dry ×20, ×40, and/or ×60 objectives. SISH signals were visualised as single copies, multiple copies, and clusters. Not every nucleus demonstrated one or two discrete small dense black signals, because a 4 μm paraffin section contains both intact and partial nuclei. In some cases, the SISH component was visualised as a confluent black nuclear signal, where individual signal enumeration was not possible. When clusters of dots representing many copies of the *HER2* gene were present, small clusters were scored as 6 copies and large clusters were scored as 12 copies.[14] Gene amplification status was reported using the conventional US Food and Drug Administration (FDA) scoring criteria (amplified if ratio $\geq$2, unamplified if ratio <2).

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 (SPSS, USA). Survival curves were plotted using the Kaplan–Meier method, and significance was determined using the log-rank test. Multivariate relationships between patient survival and biomarkers, along with other clinical parameters, were investigated using a Cox proportional hazards regression model. All p values of less than 0.05 were considered to indicate statistical significance.

RESULTS

EGFR protein expression and gene CNV

A total of 84 cases of distal EHCC were examined by EGFR immunohistochemistry. These were classified into four categories: 3+ (positive), 2+ (equivocal), 1+ (negative) and 0 (negative). Representative photomicrographs of EGFR immunostaining in tumours are shown in Fig. 1A–D. Five cases showed moderate and complete membrane staining in >10% of cancer cells, which was classified as EGFR immunoreactivity of 2+ (Fig. 1C). The other five cases demonstrated strong and complete membrane staining in >10% of cancer cells, which was defined as EGFR immunoreactivity of 3+ (Fig. 1D). FISH was performed on 21 cases, including all 3+ and 2+ category cases and randomly selected 1+ and 0 category cases. Amplification or high polysomy was observed in eight cases (Fig. 2A,B). All 3+ EGFR immunoreactivity cases showed either amplification ($n = 1$) or high polysomy ($n = 4$), and three cases (60%) with 2+ EGFR immunoreactivity showed high polysomy (Table 1). An amplification case showed tight *EGFR* gene clusters (Fig. 2A). High polysomy cases showed a striking copy number gain of the EGFR gene ($\geq$4 copies in $\geq$40% of cells), but the ratio of *EGFR* gene to the centromere was less than 2 (Fig. 2B). Two cases with EGFR immunoreactivity of 2+ demonstrated increased copy number in some tumour cells, but it did not reach 4 copies in $\geq$40% of cells; therefore, these cases were classified as having low polysomy. We defined EGFR CNV as amplification and high polysomy and positive EGFR expression as 2+ and 3+, respectively. Overall diagnostic sensitivity and specificity for predicting *EGFR* gene CNV are calculated in Table 1, and they were found to be 100% and 84.6%, respectively.

HER2 protein expression and gene CNV

HER2 immunohistochemistry for protein expression was also performed on 84 specimens of distal EHCC. These were classified into the same four scoring categories as described