Characterization of EGFR family gene aberrations in cholangiocarcinoma

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Abstract. Cholangiocarcinoma (CCA) is a highly lethal malignancy of the biliary tract with very few treatment options. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER2) have been considered as potential therapeutic targets in CCA. In the present study, we attempted to clarify the clinicopathological significance of all EGFR family members, EGFR, HER2, HER3 and HER4, across the full spectrum of CCAs. Immunohistochemistry and FISH were performed to validate expressions and genetic aberrations of these molecules retrospectively in 175 CCA patients. EGFR, HER3 and HER4 were overexpressed in 20 (30.8%), 8 (12.3%) and 41 (63.1%) of the 65 intrahepatic cholangiocarcinomas (IHCCs), and in 23 (20.9%), 13 (11.8%) and 62 (56.4%) of the 110 extrahepatic cholangiocarcinomas (EHCCs), respectively. Overexpression of HER2 was exclusively identified in EHCCs, among which the rate was 4.5% (5/110). A significant association was identified between EGFR amplification and EGFR overexpression (P=0.002). Similarly, HER2 amplification was strongly associated with HER2 overexpression (P<0.001). Multivariate analysis suggested that EGFR overexpression is an independent prognostic factor in IHCC, but not in EHCC cases [HR (95% CI): 3.689 (1.253-10.587), P=0.018]. Notably, for the first time, we demonstrated HER4 expression is a prognostic factor in EGFR-negative IHCC patients. *In vitro* data further suggested a tumor-suppressor role of HER4 in CCA. siRNA knockdown of HER4 significantly increased RBE cell migration and invasion. By contrast, HER4 overexpression decreased proliferation of HuCCT-1 cells and their migratory and invasive capacity. In summary, our results revealed expression of the EGFR family members in CCA development and progression. CCAs differentially express HER2 protein based on tumor location. HER4 expression status allows stratification of CCA patients into different survival categories.

Introduction

Cholangiocarcinoma (CCA) is a rare but highly aggressive cancer, accounting for ~3% of all gastrointestinal malignancies (1). CCA is characterized by poor responsiveness to chemotherapy and radiotherapy in the majority of cases (2,3). Surgical resection is the only potentially curative option. Therefore, novel biomarkers are urgently needed for CCA management and treatment.

The human epidermal growth factor receptor (EGFR) family consists of four members: HER1 (EGFR), HER2 (c-erbB-2), HER3 (c-erbB-3) and HER4 (c-erbB-4) (4). These receptors activate multiple downstream pathways in response to extracellular ligands, regulating diverse processes that include differentiation, migration, proliferation and survival (5,6). Aberrations in EGFR family members play a role in the development and progression of many human cancers (7-9). Extensive studies have reported either the overexpression or amplification of EGFR and HER2 in different malignancies (10,11). Accordingly, current evidence suggests that HER3 expression is associated with increased cellular proliferation whereas HER4 activation seems to mainly mediate antiproliferative effects (12-14).

In CCA, overexpression of EGFR and HER2 are thought to be prognostic factors and targets of novel biologic agents (15). Of note, a series of studies have revealed overexpression of EGFR and HER2, amplification and mutations of these genes (15-17). Although the clinical significance of such overexpression is not fully clear, some case reports and phase II trials have reported promising results targeting EGFR in CCA (18). By contrast, the data of HER3 and HER4 is very limited. Most recently, Lee *et al* (19) reported that HER3 is overexpressed in a subset of extrahepatic cholangiocarcinoma (EHCC) patients and HER3 overexpression is correlated with decreased patient survival. The biological function and prognostic role of HER4 has thus far not been investigated in CCA.

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According to the 7th edition of the Union for International Cancer Control-American Joint Committee on Cancer (UICC/AJCC), CCAs are classified as intrahepatic cholangiocarcinomas (IHCCs) and EHCCs, the latter being further divided into perihilar CCAs and distal CCAs. Although linked anatomically and histopathologically, it is unclear whether CCA patients from different sites share common pathogenetic features. It has been reported that CCA differentially expresses cell cycle-regulatory proteins based on tumor location and morphology (20).

In the present study, we comprehensively characterized and compared differential gene expression of all EGFR family members across the full spectrum of CCAs. We aimed to define differential expression of EGFR family members based on anatomic site of origin to establish new potential prognostic factors in CCA.

Patients and methods

Patients and tissue microarray (TMA) construction. The present study consisted of 175 CCAs (male, 106; female, 69) who underwent surgical resections between 2004 to 2011 at the Qilu Hospital of Shandong University (Jinan, China). Follow-up time ranges from 3 to 98 months (mean, 26 months). A total of two TMAs were constructed. Two cores (1.0 mm in diameter) were taken from each representative tumor focus and the morphology was evaluated by two pathologists (B.H. and X.Y.). Detailed clinical and pathological profiles were obtained from medical records and maintained in a secure relational database with TMA data. Tumor staging and histological classification were assessed according to AJCC 7th Edition of TNM Staging (21). Patient demographics are shown in Table I. Informed written consent was obtained from the CCA patients. The study and the consent procedures were approved by the Institutional Review Board at the School of Medicine of Shandong University. All cases were anatomically classified into two groups: IHCC and EHCC. Hilar and distal CCA were classified as EHCC. The numbers of IHCC and EHCC cases were 65 and 110, respectively.

Immunohistochemistry (IHC). IHC was performed as previously described (22). Briefly, the slides were deparaffinized by successive passages through xylene and ethanol. Antigen retrieval was performed by microwave pretreatment in 0.01 M citrate buffer (pH 6.0) for 15 min. The primary antibodies used were anti-EGFR (1:500; Dako), anti-HER2 (1:500; Dako) anti-HER3 (sc-415, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-HER4 (sc-283, 1:500; Santa Cruz Biotechnology). The slides were incubated overnight at 4˚C. For visualization, 3,3-diaminobenzidine tetrahydrochloride was used as chromogen. The slides were evaluated by two independent pathologists (B.H. and X.Y.) who were blinded to the clinical data. For EGFR and HER2, only the membrane immunostaining was scored following a four-step scale (scores 0, 1+, 2+ and 3+). For HER2, we followed the consensus panel recommendations on HER2 scoring for breast cancer (8). Slides with a score of 2+ or 3+ were classified as positive or expressed, in contrast to slices with a score of 0 or 1+, which were defined as negative. For HER3 and HER4, nuclear and cytoplasmic staining was evaluated using the Rajkumar score (23), which was built by multiplying the scores of 2 param-

Table I. Summary of CCA patient demographics.

Parameters	IHCC (%)	EHCC (%)
Age (years)		
<60	35 (53.8)	73 (66.4)
≥60	30 (46.2)	37 (33.6)
Gender		
Male	29 (44.6)	77 (70.0)
Female	36 (55.4)	33 (30.0)
Tumor size (cm)[a]		
<5	22 (33.8)	68 (61.8)
≥5	43 (66.2)	42 (38.2)
Histological differentiation		
Well	17 (26.2)	56 (50.9)
Moderate	31 (47.7)	37 (33.6)
Poor	17 (26.2)	17 (15.5)
Perineural invasion		
Negative	57 (87.7)	80 (72.7)
Positive	8 (12.3)	30 (27.3)
Microvascular invasion		
Negative	51 (78.5)	97 (88.2)
Positive	14 (21.5)	13 (11.8)
Venous invasion		
Negative	62 (95.4)	103 (93.6)
Positive	3 (4.6)	7 (6.4)
T stage		
I + II	51 (78.5)	57 (51.8)
III + IV	14 (21.5)	53 (78.2)
N stage		
Negative	50 (76.9)	78 (70.9)
Positive	15 (23.1)	32 (29.1)
UICC stage		
I + II	40 (61.5)	63 (57.3)
III + IV	25 (38.5)	47 (42.7)

[a]In EHCC tumor size was categorized by <3 and ≥3, respectively.

eters, the staining intensity (range, 0-3) and the percentage of positive cells (range, 0-4; 0, 0-10%; 1, 11-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%). Slides with scores of ≥8 were classified as overexpression and slides with scores <8 as non-overexpression.

Fluorescence in situ hybridization (FISH). FISH analysis for *EGFR* and *HER2* gene aberrations was performed as previously described (10). Briefly, the GLP *EGFR*/CSP 7 probe and GLP *HER2*/CSP17 (Beijing GP Medical Technologies, Beijing, China) were utilized and slides were examined using an ImagingZ1 microscope (Carl Zeiss, Oberkochen, Germany). FISH signals were scored manually (100x oil immersion) in morphologically intact and non-overlapping nuclei by a pathologist (B.H.) and a minimum of 50 cancer cells from each site were recorded. Cancer sites with very weak or no signals were recorded as insufficiently hybridized.
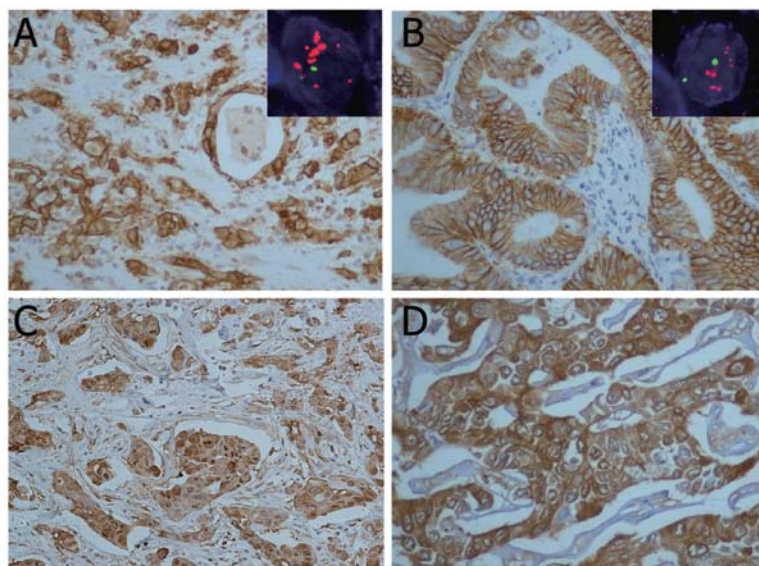
Figure 1. Representative positive cases of CCA for EGFR family members by IHC and FISH. (A and B) Strong membrane staining (score 3) of EGFR and HER2, respectively. Insert areas with representative FISH image of amplification of EGFR and HER2, respectively. (C) Strong expression of HER3 in cytoplasm and nuclei of tumor cells. (D) Strong expression of HER4 predominantly in cytoplasm of CCA cells.

A previously documented method was utilized to validate genetic aberrations of EGFR and HER2 (22). A case was scored as amplification when >10% of tumor cells displayed either definite cluster of locus probe signals or EGFR (HER2):CEP 17 ratio >2.

Cell culture and reagents. The CCA cell lines RBE, HuCCT-1, QBC939 and one breast cancer cell line MCF-7 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured following the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed as previously described (22). Briefly, the membrane was incubated overnight at 4°C with primary antibody for anti-HER4 (sc-283, 1:1,000; Santa Cruz Biotechnology). The secondary antibody was a goat anti-rabbit antibody at a dilution of 1:5,000 and the signals were detected with RapidStep™ ECL reagent (Millipore Corp., Billerica, MA, USA). Three independent experiments were performed. For analysis of the western blot images, the ImageJ software (1.37v; Wayne Rasband, NIH, Bethesda, MD, USA) was used.

In vitro overexpression of HER4. Human HER4 cDNA was subcloned into the pcDNA3.1 eukaryotic expression vectors. HER4 and empty control plasmids were independently transfected into HuCCT-1 cells using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

siRNA knockdown. siRNA transfection on CCA cell line RBE was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Three specific siRNAs for each gene were designed and synthesized by GenePharma (Shanghai, China), respectively. The most effective single siRNA-HER4 (sense strand, 5'-GCGCAGGAAA CAUCUAUAUUTT-3' and antisense strand, 5'-AUAUAGAUG GUUUCCUGCGCTT-3') was used for further experiments.

Non-specific negative control siRNAs were also used (sense strand, 5'-UUCUCCCAACGUGUCACG-3' and antisense strand, 5'-ACGUGACACGUUCGGAGAATT-3'). The mock group was defined as the ones supplemented with the transfection reagent only.

Cell proliferation, migration and invasion assays. Cell proliferation was measured by MTT assays as previously described (22). For each assay, 20 µl MTT (methyl thiazolyl tetrazolium) was added to each well and incubated for 4 h. For cell migration assay, a wound was created by a p200 pipette tip on cells grown to confluence using 6-well plates; the cell-free space was measured on images captured at both 0 and 48 h. The invasion assays were performed as previously described (22). All experiments were performed in triplicates.

Statistical analysis. The SPSS statistical software package, standard version 17.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The association between expression of EGFR family members and the clinicopathological variables was analyzed by using Chi-square test. Cumulative overall survival rates were calculated by Kaplan-Meier method and statistical significance for survival curves comparison was analyzed by log-rank test. Univariate and multivariate survival analyses were performed using the Cox multiple hazards model to estimate hazard ratio (HR) and 95% confidence interval (CI) of each outcome. Differences for all the tests were regarded as statistically significant when the P-value from a two-tailed test was $P<0.05$.

Results

Expression and genetic aberrations of EGFR family members in CCAs. Representative cases of positive staining by IHC for each member are shown in Fig. 1. EGFR, HER3 and HER4 were overexpressed in 20 (30.8%), 8 (12.3%) and 41 (63.1%) of the 65 IHCCs, and in 23 (20.9%), 13 (11.8%) and 62 (56.4%)

Table II. Association of expression of EGFR family members with clinicopathological parameters in IHCC.

Parameters	EGFR			HER3			HER4		
	Not overexpressed (%)	Overexpressed (%)	P-value	Not overexpressed (%)	Overexpressed (%)	P-value	Not overexpressed (%)	Overexpressed (%)	P-value
Age (years)									
<60	23 (65.7)	12 (34.3)		31 (88.6)	4 (11.4)		14 (40.0)	21 (60.0)	
≥60	22 (73.3)	8 (26.7)	0.507	26 (86.7)	4 (13.3)	1.000	10 (33.3)	20 (66.7)	0.579
Gender									
Male	18 (62.1)	11 (37.9)		23 (79.3)	6 (20.7)		11 (37.9)	18 (62.1)	
Female	27 (75.0)	9 (25.0)	0.262	34 (94.4)	2 (5.6)	0.143	13 (36.1)	23 (63.9)	0.880
Tumor size									
<5 cm	14 (63.6)	8 (36.4)		19 (86.4)	3 (13.6)		7 (31.8)	15 (68.2)	
≥5 cm	31 (72.1)	12 (27.9)	0.485	38 (88.4)	5 (11.6)	1.000	17 (39.5)	26 (60.5)	0.542
Histological differentiation									
Well	15 (88.2)	2 (11.8)		17 (100)	0 (0.0)		7 (41.2)	10 (58.8)	
Moderate	22 (71.0)	9 (29.0)		27 (87.1)	4 (12.9)		12 (38.7)	19 (61.3)	
Poor	8 (47.1)	9 (52.9)	0.033	13 (76.5)	4 (23.5)	0.047	5 (29.4)	12 (70.6)	0.746
Perineural invasion									
Negative	38 (66.7)	19 (33.3)		50 (87.7)	7 (12.3)		20 (35.1)	37 (64.9)	
Positive	7 (87.5)	1 (12.5)	0.432	7 (87.5)	1 (12.5)	1.000	4 (50.0)	4 (50.0)	0.669
Vascular invasion									
Negative	36 (70.6)	15 (29.4)		44 (86.3)	7 (13.7)		20 (39.2)	31 (60.8)	
Positive	9 (64.3)	5 (35.7)	0.900	13 (92.9)	1 (7.1)	0.838	4 (28.6)	10 (71.4)	0.465
T stage									
I + II	35 (68.6)	16 (31.4)		44 (86.3)	7 (13.7)		16 (31.4)	35 (68.6)	
III + IV	10 (71.4)	4 (28.6)	1.000	13 (92.9)	1 (7.1)	0.838	8 (57.1)	6 (42.9)	0.077
Lymph node metastasis									
Negative	35 (70.0)	15 (30.0)		43 (86.0)	7 (14.0)		19 (38.0)	31 (62.0)	
Positive	10 (66.7)	5 (33.3)	1.000	14 (93.3)	1 (6.7)	0.756	5 (33.3)	10 (66.7)	0.743
UICC stage									
I + II	27 (67.5)	13 (32.5)		33 (82.5)	7 (17.5)		11 (27.5)	29 (72.5)	
III + IV	18 (72.0)	7 (28.0)	0.702	24 (96.0)	1 (4.0)	0.221	13 (52.0)	12 (48.0)	0.046

of the 110 EHCCs, respectively. Overexpression of HER2 was exclusively identified in EHCCs, among which the rate was 4.5% (5/110). FISH analysis for EGFR and HER2 was available for 169 and 171 cases, respectively. In all, amplification of EGFR was identified in 1 (1.6%) of the 63 IHCC cases, comparable with that of 2.8% (3/106) in patients with EHCC. By contrast, HER2 amplification was observed in 8 of the 108 (7.4%) EHCC, but was absent in IHCC cases. Of note, a significant association was identified between EGFR amplification and EGFR overexpression (P=0.002). Similarly, HER2 amplification was strongly associated with HER2 overexpresssion (P<0.001).

Overall, overexpression of any EGFR family member was found in 47 (72.3%) of 65 patients in IHCC, and 76 (69.1%) of 110 cases in EHCC, respectively. The combination of EGFR and HER4 was the most common, found in 23.1% of IHCC tumors (n=15) and 12.7% of EHCC tumors (n=14). In contrast, none of the CCA tumors co-expressed EGFR and HER2.

Associations between EGFR family members with clinico-pathological variables. In IHCC, EGFR overexpression was significantly associated with poor histological differentiation (P=0.033), but not with age (P=0.507), tumor size (P=0.485), lymph node metastasis (P=1.000), vascular invasion (P=0.900), perineural invasion (P=0.432) or UICC stage (P=0.702) (Table II). In all, 9 EGFR-positive cases were observed in 17 (52.9%) poor differentiation samples, whereas only 11 out of 48 (22.9%) cases with well and/or moderate differentiation demon-
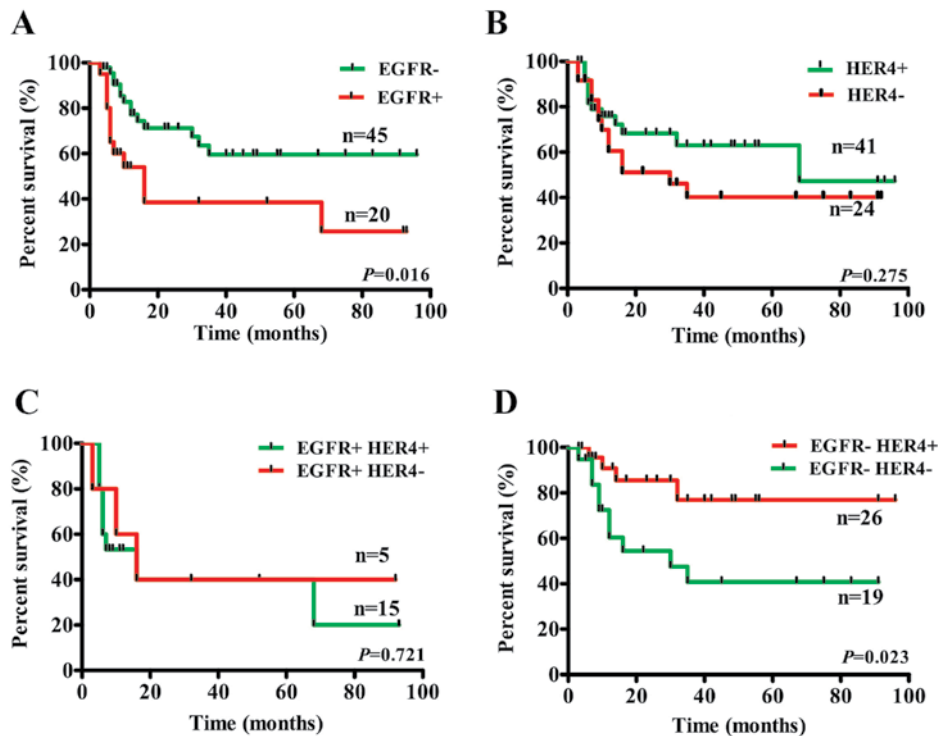
Figure 2. Survival curves stratified by EGFR and HER4 in IHCC (Kaplan-Meier method). Univariate analysis of (A) EGFR and (B) HER4 in IHCC is shown. (C) HER4 is not a prognostic factor in EGFR-positive cases. (D) The outcome of HER4-positive cases was significantly better than that of HER4-negative cases in EGFR-negative IHCCs.

strated EGFR positivity. Poorly differentiated tumors were significantly associated with HER3 overexpression (P=0.047). By contrast, HER4 expression was associated with UICC I/II stage (P=0.046), but not with well differentiation (P=0.746). No significant association of EGFR amplification was identified with tumor size, histological type, clinical stage, presence of vascular or perineural invasion or lymph node metastases.

In EHCC, EGFR overexpression was significantly associated with well histological differentiation (P<0.001) and lymph node metastasis (P=0.006). Overexpression of HER2 was significantly associated with TI/TII stage (P=0.050), but not with histo-logical differentiation or lymph node metastasis (Table III). No significant correlation was identified between HER3 or HER4 overexpression with other clinicopathological factors.

Univariate and multivariate analyses in CCA. In IHCC, univariate analysis revealed that EGFR overexpression was a prognostic factor (P=0.016). Additionally, tumor size (P=0.022) and lymph node metastasis (P<0.001) were also significantly related to overall survival. Notably, in a multivariate analysis, EGFR overexpression remained an independent prognostic factor [HR (95% CI): 3.689 (1.253-10.587), P=0.018] (Table IV).

In EHCC, 4 factors including EGFR overexpression were identified as prognostic factors by univariate analysis. In multivariate analysis, as shown in Table V, only lymph node status was an independent prognostic factor [HR (95% CI): 2.429 (1.120-5.266), P=0.025]. In contrast, EGFR expression lost its predictive value.

Overall, no statistical significance was identified between HER4 expression and overall survival in CCA by univariate analysis. However, HER4 expression was identified as a

prognostic factor (P=0.023) in EGFR-negative IHCC cases (Table VI). While in EGFR-positive cases, HER4 expression showed no influence on survival rate (P=0.721) (Fig. 2). In EHCC, no significant correlation was present between HER4 expression and overall survival both in EGFR+ and EGFR- cases (data not shown).

Due to a very limited number of HER2-positive cases, survival analysis was not performed for HER2 expression either in univariate or multivariate analysis.

HER4 inhibits cellular proliferation, migration and invasion in CCA cell lines. Using the MTT assay, we found that siRNA knockdown of HER4 in RBE cells significantly increased cell proliferation at 24 and 48 h after treatment compared with negative controls (n=3, P<0.05; Fig. 3). Wound healing assay indicated that siRNA-HER4-transfected RBE cells displayed a significant decrease in cell migration ability compared to control conditions (P<0.05; Fig. 3). To further examine the effect of HER4 on cell invasion, siHER4-transfected RBE and PcDNA3.1-HER4-transfected HuCCT-1 cells were cultured in a Transwell apparatus. The percentage of migrated cells was significantly less in siHER4-treated groups when compared to the negative control groups (for both P<0.05). PcDNA3.1-HER4-transfected HuCCT-1 cell line showed increased percentage of migrated cells (both P<0.05; Fig. 3).

Discussion

To the best of our knowledge, this is the largest cohort of CCA patients reported thus far, investigating aberrations of all four EGFR family members. Previously, only one

Table III. Association of expression of EGFR family members with clinicopathological parameters in EHCC.

Parameters	EGFR			HER2			HER3			HER4		
	Not overexpressed (%)	Overexpressed (%)	P-value	Not overexpressed (%)	Overexpressed (%)	P-value	Not overexpressed (%)	Overexpressed (%)	P-value	Not overexpressed (%)	Overexpressed (%)	P-value
Age (years)												
<60	59 (80.8)	14 (19.2)		68 (93.2)	5 (6.8)		64 (87.7)	9 (12.3)		29 (39.7)	44 (60.3)	
≥60	28 (75.7)	9 (24.3)	0.531	37 (100)	0 (0.0)	0.252	33 (89.2)	4 (10.8)	0.889	19 (51.4)	18 (48.6)	0.245
Gender												
Male	63 (81.8)	14 (18.2)		73 (94.8)	4 (5.2)		69 (89.6)	8 (10.4)		35 (45.5)	42 (54.5)	
Female	24 (72.7)	9 (27.3)	0.283	32 (97.0)	1 (3.0)	1.000	28 (84.8)	5 (15.2)	0.698	13 (39.4)	20 (60.6)	0.557
Tumor size (cm)												
<3	52 (77.6)	16 (22.4)		63 (92.6)	5 (7.4)		63 (92.6)	5 (7.4)		30 (44.1)	38 (55.9)	
≥3	35 (83.3)	7 (16.7)	0.390	42 (100)	0 (0.0)	0.184	34 (81.0)	8 (19.0)	0.123	18 (42.3)	24 (57.1)	0.897
Histological differentiation												
Well	53 (94.6)	3 (5.4)		54 (96.4)	2 (3.6)		51 (91.1)	5 (8.9)		26 (46.4)	30 (53.6)	
Moderate	26 (70.3)	11 (29.7)		34 (91.9)	3 (8.1)		32 (86.5)	5 (13.5)		15 (40.5)	22 (59.5)	
Poor	8 (47.1)	9 (52.9)	<0.001	17 (100)	0 (0.0)	0.453	14 (82.4)	3 (17.6)	0.688	7 (41.2)	10 (58.8)	0.834
Perineural invasion												
Negative	63 (78.8)	17 (21.3)		77 (96.3)	3 (3.8)		70 (87.5)	10 (12.5)		34 (42.5)	46 (57.5)	
Positive	24 (80.0)	6 (20.0)	0.886	28 (93.3)	2 (6.7)	0.889	27 (90.0)	3 (10.0)	0.889	14 (46.7)	16 (53.3)	0.695
Vascular invasion												
Negative	77 (79.4)	20 (20.6)		92 (94.8)	5 (5.2)		88 (90.7)	9 (9.3)		40 (41.2)	57 (58.8)	
Positive	10 (76.9)	3 (23.1)	1.000	13 (100)	0 (0.0)	1.000	9 (69.2)	4 (30.8)	0.072	8 (61.5)	5 (38.5)	0.166
T stage												
I + II	42 (80.8)	10 (19.2)		47 (90.4)	5 (9.6)		46 (88.5)	6 (11.5)		22 (42.3)	30 (57.7)	
III + IV	45 (77.6)	13 (22.4)	0.682	58 (100)	0 (0.0)	0.050	51 (87.9)	7 (12.1)	0.833	26 (44.8)	32 (55.2)	0.790
Lymph node metastasis												
Negative	67 (85.9)	11 (14.1)		73 (93.6)	5 (6.4)		71 (91.0)	7 (9.0)		31 (39.7)	47 (60.3)	
Positive	20 (62.5)	12 (37.5)	0.006	32 (100.0)	0 (0.0)	0.336	26 (81.3)	6 (18.7)	0.264	17 (53.1)	15 (46.9)	0.199
UICC stage												
I + II	49 (77.8)	14 (22.2)		58 (92.1)	5 (7.9)		59 (93.6)	4 (6.4)		26 (41.3)	37 (58.7)	
III + IV	38 (80.9)	9 (19.1)	0.695	47 (100)	0 (0.0)	0.130	38 (80.8)	9 (19.2)	0.078	22 (46.8)	25 (53.2)	0.562

Table IV. Univariate and multivariate analysis of variables associated with survival in IHCC patients.

Variables	Coding	Univariate analysis	Multivariate analysis	
			HR (95% CI)	P-value
Tumor size	<5 vs. ≥5	0.012	3.571 (1.524-8.370)	0.003
Lymph node metastasis	Neg vs. pos	<0.001	4.248 (1.761-10.249)	0.001
EGFR IHC	Neg vs. pos	0.016	3.689 (1.253-10.587)	0.018

Neg, negative; pos, positive.

Table V. Univariate and multivariate analysis of variables associated with survival in EHCC patients.

Variables	Coding	Univariate analysis	Multivariate analysis	
			HR (95% CI)	P-value
Histological differentiation	Well/moderate vs. poor	<0.001	Non significant	
pT stage	I + II vs. III + IV	0.006	Non significant	
Lymph node metastasis	Neg vs. pos	<0.001	2.429 (1.120-5.266)	0.025
EGFR IHC	Neg vs. pos	0.026	Non significant	

Neg, negative; pos, positive.

Table VI. Univariate analysis of HER4 expression in patients with IHCC stratified by EGFR.

	IHCC		
	N	Survival %	P-value
EGFR-			
HER4-	19	47.4	
HER4+	26	84.6	0.023
EGFR+			
HER4-	5	40.0	
HER+	15	40.0	0.721

study reported expression and clinical significance of all EGFR family members in a small cohort (n=56) of IHCC (24). In the present study, we firstly confirmed a strong link of EGFR to the biological aggressiveness in Chinese CCA patients. The prevalence of EGFR overexpression in CCA has been presented in various reports, ranging from 8.1 to 81% (15,17,25,26). This broad range might be explained by the lack of a standardized methodology, different standards of interpretation or differences in tumor location. Similar to Yoshikawa *et al* (15), we detected the frequency of EGFR aberration in our cohort was 30.8% in IHCC and 20.9% in EHCC cases. The EGFR overexpression is linked to poor histological differentiation and lymph node metastasis. More importantly, EGFR expression is an independent prognostic indicator in Chinese patients with IHCC. In a systematic review of prognostic biomarkers in CCA, Ruys *et al* (27)

showed that EGFR, along with other biomarkers fascin, MUC1, MUC4 and p27 is associated with survival in patients with resected CCA. To date, EGFR expression in CCA makes it a promising target for EGFR-directed therapy. Preliminary studies suggest that combination chemotherapy using anti-EGFR antibody, along with conventional therapeutic agents, increase the efficacy of treatment (28). In a recent clinical phase II trial, Chiorean *et al* (29) suggested that anti-EGFR therapy remained an important option in advanced biliary cancers but only with a molecular 'targeted' approach.

In the present study, another key finding was that HER4 expression is a prognostic factor in EGFR-negative IHCC cases. To date, the role of HER4 expression in cancer remains controversial. Some studies demonstrated that HER4 acts as a tumor suppressor in aggressive cancers such as the breast, prostate, pancreas and larynx (8,30-33). HER4 expression is antiproliferative and clinically associated with increased survival, reduced recurrence, as well as antagonizing the effect of HER2. By contrast, some reports suggested HER4 overexpression in medulloblastomas and ependymomas possesses oncogenic activities (34,35). In the present study, we provided several lines of evidence to show the antitumor role of HER4 expression in CCA. Firstly, HER4 expression seems to be related to earlier clinical stage and lower tendency of lymph node metastasis, although these did not reach significant difference. Secondly, *in vitro* data demonstrated that HER4 knockdown in RBE cells promotes cellular proliferation, motility and invasion. Thirdly, a prognostic role of HER4 expression was identified in a subset of IHCC cases. These findings reinforce the importance of molecular subclassification (whether through HER4 status or other relevant biomarkers) of EGFR-negative CCA patients in clinical trials.
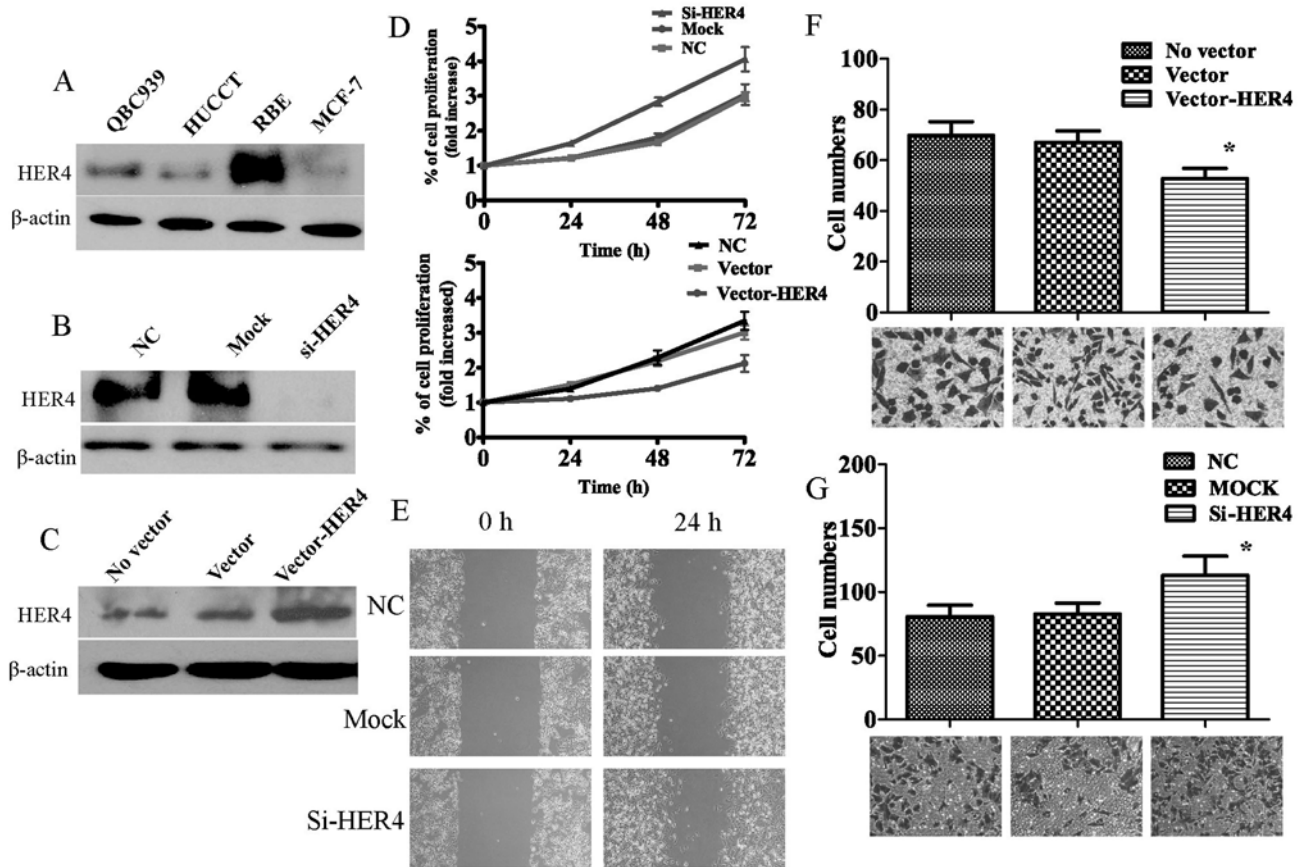
Figure 3. HER4 expression inhibits cellular proliferation, migration and invasion of CCA cells. (A) The protein expression level of HER4 in QBC939, HuCCT-1, RBE and breast cancer cell line MCF-7. (B) HER4 protein expression level after siRNA knockdown of HER4 in RBE cells. (C) HER4 protein expression in pcDNA3.1-HER4 transfected HuCCT-1 cells. (D) Cell proliferation ability assessed by MTT assay in RBE (above) and HuCCT-1 (below), respectively. (E) Effects of siRNA HER4 on RBE cell migration by wound healing assay. Original magnification, x100. (F and G) Cellular invasive capacity of CCA cells assessed by invasion chamber assays; upper, siRNA knockdown of HER4 in RBE cells; lower, pcDNA3.1-HER4 in HuCCT-1 cells. The data shown are the means ± SD of triplicate experiments; *P<0.05.

The reason why HER4 expression was not a prognostic factor in EHCC might be partially explained by variables associated with their anatomic behavior and methods of surgery. Our data suggested HER4 might play a tumor suppressor role in a selected subset of Chinese CCA patients.

One aim of the present study was to examine the entire spectrum of CCA to define anatomic site-related similarities and differences. Of note, the frequencies of overexpression of EGFR, HER3 and HER4 in EHCC are similar to those of IHCC cases. However, we found that both HER2 overexpression and HER2 amplification were exclusively identified in EHCC cases. Overall, HER2 overexpression and amplification has been found in a range between 5 and 76% in CCA (16,24,25,36). Some authors suggested that HER2 overexpression is due to gene deregulation rather than gene amplification as in some studies there is no strict correlation between protein expression and gene amplification (37). However, consistent with Harder *et al* (11), our data suggested that in CCA cases with high HER2 expression there is also a good correlation between overexpression and amplification. Considering the lack of correlation between HER2 overexpression with other clinical variables, out data suggested that HER2 overexpression might play a role in a subset of EHCC cases, but has limited significance in the development and progression of CCA.

HER3 overexpression was present in 12.3% (8/65) of IHCC and 11.8% (13/110) of EHCC. The prevalence in this study is significantly lower than almost all of those in previous studies (19,24). HER3 overexpression has been reported to be associated with a poorer outcome in melanomas (38) and lung and gastric cancers (38-40). In CCA, Lee *et al* (19) suggested that HER3 overexpression is correlated with decreased patient survival and represents a prognostic indicator of patients with EHCC. Notably, they observed the co-overexpression of HER2 and HER3 in subsets of EHCC patients. However, HER3 expression did not show significant prognostic value in the present study. Thus, the exact biological role of HER3 in CCA merits further investigation.

In summary, our results revealed expression of the EGFR family members in CCA development and progression. CCAs differentially express HER2 protein based on tumor location. For the first time, our study suggested that HER4 is a prognostic factor in subset of EGFR-negative CCA patients with IHCC. Determination of HER4 expression status allows stratification of CCA patients into different survival categories.

Acknowledgements

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