Expression of EGFRvIII and its co-expression with wild-type EGFR, or putative cancer stem cell biomarkers CD44 or EpCAM are associated with poorer prognosis in patients with hepatocellular carcinoma

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Abstract. The aberrant expression of HER family members and cancer stem cells (CSCs) have been associated with tumour progression and resistance to therapy. At present, several HER inhibitors have been approved for the treatment of patients with a range of cancers but not for the treatment of patients with hepatocellular carcinoma (HCC). The present study investigated the co-expression and prognostic significance of HER family members, type-III deletion mutant EGFR (EGFRvIII), and the putative CSC biomarkers CD44 and epithelial cell adhesion molecule (EpCAM) in 43 patients with HCC. The relative expression of these biomarkers was determined using immunohistochemistry. At a cut off value of >5% of tumour cells stained for these biomarkers, 35% [wild-type (wt)EGFR], 58% (HER-2), 0% (HER-3), 19% (HER-4), 26% (EGFRvIII), 40% (CD44) and 33% (EpCAM) of patients were positive. In 23, 14 and 9% of the patients, wtEGFR expression was accompanied by co-expression with HER-2, EGFRvIII and HER-2/EGFRvIII, respectively. EGFRvIII expression, membranous expression of CD44 and co-expression of wtEGFR/EGFRvIII were associated with poor overall survival (OS). By contrast, cytoplasmic CD44 expression was associated with a longer OS time. The present study also investigated the effect of several agents targeting one or more members of the HER family, other growth factor receptors and cell signalling proteins on the proliferation of HCC cell lines. Among agents targeting one or more members of the HER family, the pan-HER family blocker afatinib was the most effective, inhibiting the proliferation of three out of seven human liver cancer cell lines (LCCLs), while the CDK inhibitor dinacicilib was the most effective agent, inhibiting the proliferation of all human LCCLs tested. Taken together, the present results suggested that EGFRvIII expression and its co-expression with wtEGFR or CD44 was of prognostic significance. These results also support further investigations of the therapeutic potential of drugs targeting EGFRvIII and other members of the HER family in patients with HCC.

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Abbreviations: EGFRvIII, type-III deletion mutant EGFR; HCC, hepatocellular carcinoma; CSC, cancer stem cell; TKIs, tyrosine kinase inhibitors

Key words: HCC, HER family, EGFRvIII, prognosis, cyclin-dependent kinases, CD44, TKIs

Introduction

Despite preventive measures and advances in the diagnosis and treatment of cancer, liver cancer is the third leading cause of cancer-associated deaths worldwide, and hepatocellular carcinoma (HCC), which is the most aggressive type of primary liver cancer, accounts for nearly 90% of liver cancer cases (1,2). In the USA in 2024, it is estimated that 41,630 individuals will be diagnosed with cancer of the liver and intrahepatic bile duct and 29,840 individuals will die of such cancers (3).

Currently, the treatment options for HCC vary and include surgery, chemotherapy, radiotherapy, targeted therapies using multiple kinase inhibitors sorafenib, regorafenib, lenvatinib, cabozantinib, ramucirumab and bevacizumab, and treatment with checkpoint inhibitors such as ipilimumab, nivolumab, pembrolizumab and atezolimimab (2,4). While patients

identified at the early stage of the disease can be cured with surgery and local ablation, numerous patients are diagnosed at a more advanced stage of the disease and consequently do not gain a long-term benefit from such therapeutic interventions alone due to primary and secondary resistance to other treatment options (4-7). The heterogeneous nature of HCC (intra-tumour and inter-tumour heterogeneity), presence of cancer stem cells (CSCs), aberrant expression and mutation of heterologous growth factor receptor signalling pathways, epithelial-to-mesenchymal transition, and differences in the tumour microenvironment have been highlighted as some of the factors contributing to no response or a response of short duration to the current therapeutic interventions (8-13). To reduce mortality as a result of liver cancer, it is considered essential to identify additional biomarkers, to determine novel therapeutic targets and to develop more effective therapeutic agents. These may be used in the early diagnosis of HCC, in determining cancer prognosis and in predicting the response to therapy. Furthermore, it is important to identify novel therapeutic targets and to develop more effective therapeutic approaches. This may be achieved by investigation of the therapeutic application of drugs that have already been approved for other cancer types by repurposing such drugs.

Since the early 1980s, increased expression, amplification or mutation of human EGFR, which is the prototype of the type I growth factor receptor family (also called HER) with tyrosine kinase activity, have been reported in a wide range of solid tumours and these have been associated with poor prognosis in some patients (14,15). The HER family, in addition to EGFR, contains three additional members: HER-2, HER-3 and HER-4. The binding of ligand to the external domain of EGFR results in homodimerization or heterodimerization with other members of the HER family, phosphorylation of the tyrosine kinase domain of each receptor and ultimately activation of downstream cell signalling pathways, including the RAS/MAP, PI (3)K/AKT, phospholipase C (PLC)γ/PLC and Janus kinase/STAT signalling pathways (14,15). To date, several monoclonal antibodies (mAbs) and small molecules tyrosine kinase inhibitors (TKIs) targeting one or more members of the HER family have been approved for the treatment of patients with a range of cancer types (14-17). However, the clinical benefit gain may be short in some patients and no HER inhibitor has been approved for the treatment of patients with liver cancer at present (18). Furthermore, to the best of our knowledge, there is currently no comprehensive study of the relative expression of all members of the HER family and the type-III deletion mutant EGFR (EGFRvIII), which is the most common variant of EGFR containing deletion in the extracellular domain of EGFR, in patients with HCC.

Therefore, to the best of our knowledge, the present study is the first to examine the relative expression and prognostic significance of all members of the HER family, EGFRvIII, two putative liver CSC markers [CD44 and epithelial cell adhesion molecule (EpCAM)] and the tumour proliferation biomarker Ki67 in patients with HCC. Furthermore, the present study investigated the effect of various agents targeting one or more members of the HER family and CDK inhibitors compared with the Food and Drug Administration (FDA)-approved sorafenib and regorafenib on the proliferation of a panel of HCC cell lines.

Materials and methods

Patients. The present retrospective study included tumour specimens from a total of 43 patients with HCC treated between 2010 and 2019 at the Royal Surrey Hospital (Guilford, UK). The median age of the patient population was 70 years, ranging between 45 and 86 years. The ethical approval for the study was obtained from the NHS Research Ethics Committee (IRAS Project ID 252931; UK). Tumour samples from patients with insufficient tumour and no follow-up information were excluded from the study. Data on the clinicopathological features of each participant, including patient age, sex, tumour grade, differentiation, and hepatitis B and C status, were collected. All participant information was analysed anonymously.

Immunohistochemistry and scoring criteria. Immunohistochemical analysis of 43 paraffin-embedded HCC samples was performed using the Ultra View DAB kit and the Ventana Discovery Ultra autostainer (Roche Diagnostics, Ltd.) as described previously (18,19). Serial sections of tumour specimens were cut and stained with antibodies specific for various biomarkers. The co-expression levels were also determined based on whether the same tumour specimen was positive for two or more biomarkers. The primary antibodies employed in this study were: EGFRvIII (1:500; cat. no. NBP2-50599; Novus Biologicals, Ltd.; Bio-Techne), wtEGFR (1:250; DAK-H1-WT; cat. no. M7298; Dako; Agilent Technologies, Inc.), HER-2 (3B5; 1:200; cat. no. sc-33684; Insight Biotechnology), HER-3 (1:50; cat. no. ab93739; Abcam), HER-4 (1:100; cat. no. sc-53280; Insight Biotechnology), CD44 (1:40; cat. no. M7082; Agilent Technologies, Inc.), EpCAM (1:100; cat. no. NB600-1182; Novus Biologicals, Ltd.; Bio-Techne) and Ki67 (1:100; cat. no. M7240; Agilent Technologies, Inc.). All samples were scored independently by two observers including a consultant histopathologist. If any discrepancy occurred between the reviewers, the slides were re-examined. Briefly, the slides were scored based on the following characteristics as described previously (19,20): Intensity (negative, 0; weak, 1+; moderately positive, 2+; and strongly positive, 3+), location (membrane, cytoplasm or nucleus) and percentage of positively stained tumour cells (>5, >10, >20 and >50%).

Flow cytometry and determination of proliferation of liver cancer cells following treatment with various targeted agents. The expression levels of HER family members, including insulin-like growth factor 1 receptor (IGF IR; 4 μg/ml; cat. no. MAB1120; Merck KGaA), cMET (1:1,000; cat. no. ab237711; Abcam) and CD44 (10 μg/ml; cat. no. 555476; Becton, Dickinson and Company), were determined in a panel of human liver cancer cell lines (LCCLs) by flow cytometry as described previously (21). SNU-475 (ATCC CRL-2236), C3A (ATCC CRL-3581), SNU-449 (ATCC CRL-2234), PLC/PRF/5 (ATCC CRL-8024), SNU-387 (ATCC CRL-2237) and SNU-423 (ATCC CRL-2238) cells were purchased from American Type Culture Collection, and HepG2 cells were purchased from the UK Health Security Agency (cat. no. 85011430). The EGFR-overexpressing head neck cancer cell line (HN5), HER-2-overexpressing ovarian cancer cell line (Skov3) and CD44-overexpressing colorectal

Table I. Clinicopathological features of patients with hepatocellular carcinoma.

Characteristics	No. of patients (%)	Overall survival, months (mean ± SE)	95% CI	P-value
Age, years				0.02
≥65	34 (79.10)	44.00±13.64	17.26-70.74	
<65	9 (20.90)	79.99±6.13	63.98-88.04	
Sex				0.63
Male	31 (72.10)	71.86±6.53	59.07-84.65	
Female	12 (27.90)	66.49±14.61	37.86-95.12	
Tumour size, mm				0.79
≤20	2 (4.70)	69.50±31.50	7.76-131.24	
≤50	22 (51.10)	63.97±8.52	47.26-80.68	
>50	19 (44.20)	79.33±8.50	62.68-95.98	
Focal status				0.26
Unifocal	33 (76.70)	72.61±6.40	60.07-85.15	
Multifocal	10 (23.30)	62.08±15.37	31.96-92.21	
Hepatitis B				0.61
Positive	3 (6.98)	67.67±22.73	32.15-53.85	
Negative	40 (93.02)	53.10±5.85	38.40-57.60	
Hepatitis C				0.61
Positive	1 (2.33)	42.00±0.00	42.00-42.00	
Negative	42 (97.67)	54.41±5.75	43.14-65.67	

Overall survival was compared between groups based on clinical features using Kaplan-Meier analysis and the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

cancer cell line (Caco-2), used in our previous study, were used as positive controls for these biomarkers in the present study (22).

All cell lines were grown in an incubator set at 5% CO_2 in medium containing 10% FBS (Sigma-Aldrich; Merck KGaA) enriched media with addition of antibiotics, including penicillin (50 μg/ml), streptomycin (50 μg/ml) and neomycin (50 μg/ml) (Sigma-Aldrich; Merck KGaA). The HEP-G2, PLC/PRF/5, C3A (HepG2/C3A, derivative of Hep G2), Caco-2, SKOV3 and HN5 cell lines were grown in DMEM (Sigma-Aldrich; Merck KGaA). SNU 449, SNU 475, SNU 387 and SNU 423 cells were all grown in RPMI-1640 Medium (Sigma-Aldrich; Merck KGaA). Furthermore, 2 mM L-Glutamine (Sigma-Aldrich; Merck KGaA) was added to the DMEM and RPMI-1640 medium.

The proliferation of a panel of human LCCLs following treatment with various agents targeting different members of the HER family, other growth factor receptors and downstream cell signalling molecules was also determined using a sulforhodamine B assay as described previously (21).

Statistical analysis. Statistical assessments were carried out using SPSS software (SPSS statistics version 26; IBM Corp.). Pearson's χ^2 test and Fisher's exact test were used to determine the association between clinicopathological features and immunohistochemistry scores. Kaplan-Meier curves were used to determine if there was a significant association between biomarker expression and overall survival (OS; months). Cox regression analysis was performed to determine whether the biomarkers were significantly associated with OS and whether they were an independent factor based on univariate and multivariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of patients with HCC. The clinicopathological features and their associations with OS are presented in Table I. The mean OS time of all patients was 60.03 months. Age was significantly associated with OS (P=0.02). Patients >65 years old had an average OS time of 44.00 months, while those <65 years old had an OS time of 79.99 months. No association was observed between the other characteristics and OS (Table I).

Expression levels of HER family members in patients with HCC. To the best of our knowledge, the present study was the first to determine the expression of all HER family members and EGFRvIII in tumour specimens from patients with HCC. At the cut off value of >5% of tumour cells with positive staining, 35% of the cases were EGFR-positive. The cellular location of EGFR staining was membranous and cytoplasmic in 33 and 12% of patients, respectively. None of the staining was present at an intensity of 3+; however, 30 and 7% of the patients had an EGFR staining intensity of 1+ and 2+, respectively (Table II). At the same cut off value, 58% of patients

Table II. Expression, cellular location and intensity of the HER family members, CD44, EpCAM and Ki67 in hepatocellular carcinoma.

	Number of positive samples (%)							
Variable	wtEGFR	HER-2	HER-3	HER-4	EGFRvIII	CD44	EpCAM	Ki67
Positively stained tumour cells (%)								
>5	15 (35)	25 (58)	-	8 (19)	11 (26)	17 (40)	14 (33)	29 (67)
>10	11 (26)	24 (56)	-	3 (7)	4 (9)	13 (30)	8 (19)	21 (49)
>20	7 (16)	21 (49)	-	3 (7)	3 (7)	10 (23)	7 (16)	14 (33)
>50	4 (9)	12 (28)	-	1 (2)	1 (2)	3 (7)	1 (2)	7 (16)
Cellular location								
Membranous	14 (33)	6 (14)	-	-	-	11 (26)	3 (7)	-
Cytoplasmic	5 (12)	25 (58)	-	7 (16)	10 (23)	6 (14)	12 (28)	-
Nuclear	-	-	-	2 (5)	1 (2)	-	3 (7)	29 (67)
Intensity								
1+	13 (30)	17 (40)	-	8 (19)	11 (26)	15 (35)	10 (23)	-
2+	3 (7)	17 (40)	-	-	-	2 (5)	5 (12)	-
3+	-	5 (12)	-	-	-	1 (2)	-	-

EGFRvIII, type-III deletion mutant EGFR; EpCAM, epithelial cell adhesion molecule; wt, wild-type.

were HER-2 positive, with the cellular location of staining being membranous (14%) and cytoplasmic (58%), and with 12% of patients having strong immunostaining intensity. While 19% of the cases were HER-4 positive, none were HER-3 positive. Furthermore, 26 and 9% of the patients were EGFRvIII-positive, when the staining was scored at the cut off value of >5 and >10%, respectively, of tumour cells with positive staining (Table II; Fig. 1). The results of immunostaining for these biomarkers at other cut off values of >10, 20 and 50% are presented in Table II and images of positively stained tumour cells are presented in Fig. 1.

Expression levels of the CSC markers CD44 and EpCAM, and Ki67 in patients with HCC. The presence of a subset of cancer cells with stem cell properties has been associated with cancer recurrence and resistance to therapeutic agents, and two putative biomarkers for CSCs in HCC are CD44 and EpCAM (23). Therefore, the expression levels of these two biomarkers, as well as the cell proliferation marker Ki67, in tumour specimens were also determined. At a cut off value of >5% of tumour cells with staining, 26 and 14% of the cases exhibited membranous and cytoplasmic expression of CD44, with 5 and 35% of the cases having an immunostaining intensity of 2+ and 1+, respectively (Table II; Fig. 1). At the same cut off value, 33% of patients were EpCAM-positive, with the cellular location of staining being membranous (7%), cytoplasmic (28%) and nuclear (7%) (Table II; Fig. 1). Finally, at the same cut off value, 67% of cases exhibited Ki67 staining in the nucleus (Table II; Fig. 1).

However, at a higher cut off value of >10% of tumour cells with positive staining, 30 and 19% of patients were CD44-positive and EpCAM-positive, respectively (Table II). The cellular location of CD44 staining was membranous and cytoplasmic in 21 and 9% of patients, respectively (Fig. 1). At the same cut off value of >10, 19 and 49% of patients were EpCAM-positive and Ki67-positive, respectively, with all staining for Ki67 being nuclear (Table II). The results of staining for these biomarkers at higher cut off values are summarized in Table II.

Co-expression of the HER family members, EGFRvIII, CD44, EpCAM and Ki67 in patients with HCC. Tumour heterogeneity is common in patients with HCC. While the expression levels and prognostic significance of individual markers in patients with HCC have been assessed previously, to the best of our knowledge, there has been no study investigating the co-expression of all these biomarkers and their clinical significance (24). The examination of co-expression of HER family members may reveal the potential of cross-talk between different members of the HER family in tumour progression and consequent poor response to therapy. The identification of such cross-talk may lead to novel more effective therapeutic interventions using a cocktail of mAbs, small molecule TKIs and other therapeutic agents (25,26). The results of co-expression analysis of two or more of the biomarkers at cut off values of both >5 and >10% are presented in Table SI. At the cut off value of >5% of the tumour cells stained, 23% of the patients exhibited co-expression of wtEGFR/HER-2. In 14, 14 and 2% of the cases examined, EGFRvIII was co-expressed with wtEGFR, HER-2 and HER-4, respectively. However, the co-expression of all three members of the HER family (EGFR/HER-2/HER-4) was found to be uncommon and occurred in only 2% of the cases examined (Table SI). Next, the present study examined the co-expression of one or more members of the HER family with the CSC markers CD44 and EpCAM, and the tumour cell proliferation marker Ki67, and the results are summarized in Table SI. For example,

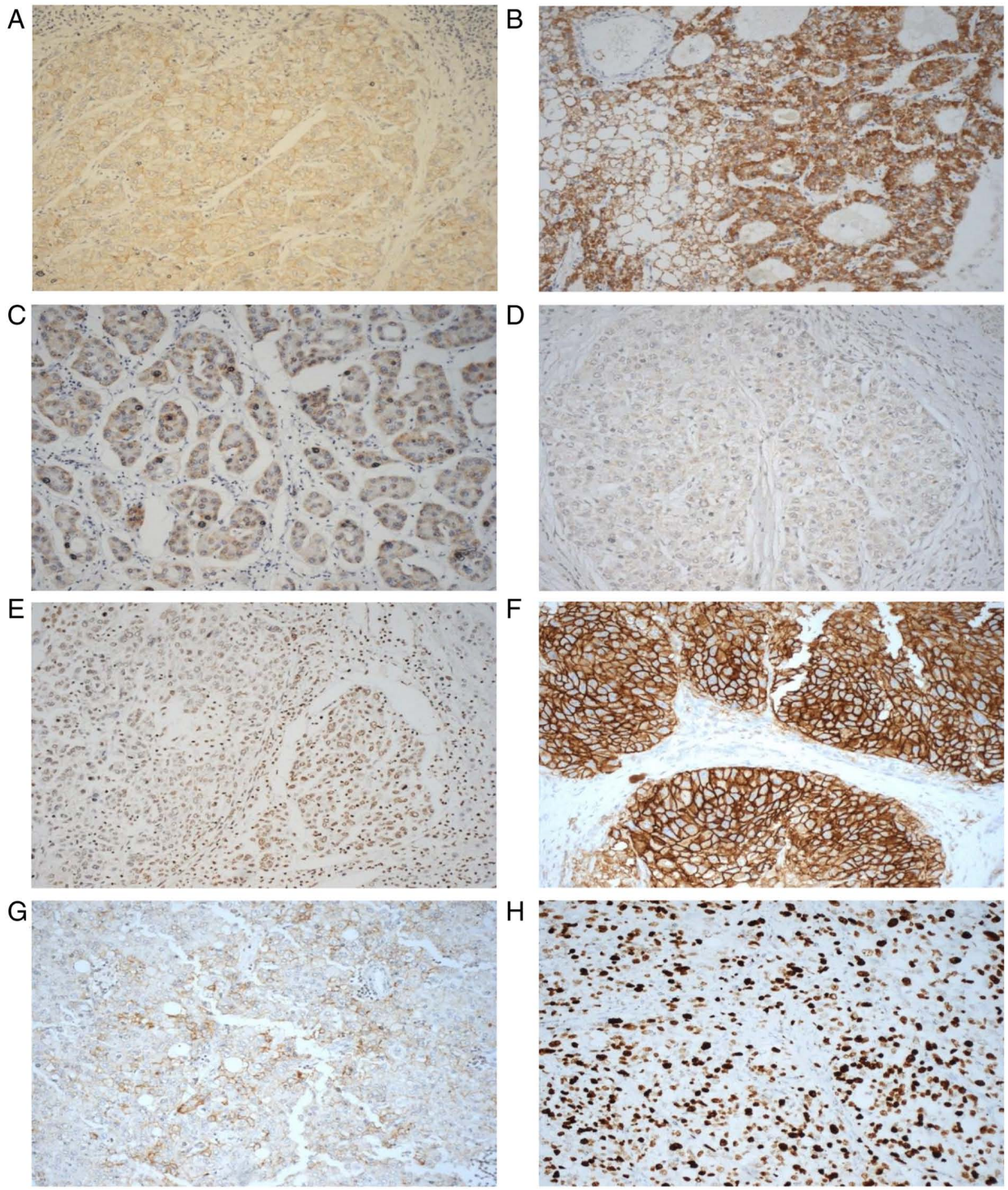

Figure 1. Immunohistochemical examination of hepatocellular carcinoma tumour specimens from patients to determine the expression of HER family members, EGFRvIII and cancer stem cell markers (magnification, x200). Liver cancer samples were stained using the Ventana Discovery Ultra autostainer. (A) Wild-type EGFR 1+ (membranous). (B) HER-2 3+ (cytoplasmic and membranous). (C) HER-4 1+ (cytoplasmic). (D) EGFRvIII 1+ (cytoplasmic). (E) EGFRvIII 1+ (nuclear). (F) CD44 3+ (membranous). (G) Epithelial cell adhesion molecule 2+ (membranous). (H) Ki67 >80% of cells.

at the cut off value of >5% of tumour cells with positive immunostaining, CD44 expression was accompanied by co-expression of wtEGFR (14%), HER-2 (26%), HER-4 (9%), EGFRvIII (7%), EpCAM (14%), Ki67 (30%) and Ki67/HER-2 (21%) (Table SI). At the same cut off value of >5% of tumour cells with positive staining, co-expression of EpCAM with EGFR, HER-2, HER-4 and Ki67 was present in 14, 23, 5 and 21% of the cases examined, respectively. These results and those at a cut off value of >10% of tumour cells with immunostaining are presented in Table SI.

Association of the expression of the HER family members, CD44, EpCAM and Ki67 with clinicopathological features and OS in patients with HCC. Following the determination of the expression pattern of the HER family members, the present study investigated the associations between the expression of

Table III. Association between the expression and co-expression of various biomarkers and overall survival in univariate and multivariate analysis in patients with hepatocellular carcinoma.

	Overall survival					
	Univariate analysis			Multivariate analysis		
Expression of biomarker	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
HER-2 1+ staining	0.51	0.27-0.98	0.04	0.43	0.22-0.84	0.01
EGFRvIII >5%	3.35	1.53-7.31	0.002	3.41	1.55-7.50	0.002
EGFRvIII >10%	3.13	1.07-9.15	0.04	-	-	NS
EGFRvIII >20%	7.22	1.94-26.92	0.003	7.66	2.00-29.36	0.03
Membranous CD44	2.12	1.04-4.36	0.04	2.15	1.02-4.53	0.04
Cytoplasmic CD44	0.30	0.11-0.81	0.02	0.20	0.07-0.59	0.003
EGFRvIII + wtEGFR	3.57	1.42-8.96	0.01	3.36	1.33-8.52	0.01
EGFRvIII + CD44	4.03	1.17-13.91	0.03	3.97	1.15-13.67	0.03
EGFRvIII+ EpCAM	2.68	1.08-6.68	0.03	-	-	NS
EGFRvIII + Ki67	2.73	1.20-6.22	0.02	3.28	1.41-7.62	0.01
EGFRvIII + wtEGFR + Ki67	3.38	1.27-9.02	0.02	3.81	1.41-10.30	0.01

P≤0.05 was considered to indicate a statistically significant difference (cut off score >5%). EGFRvIII, type-III deletion mutant EGFR; EpCAM, epithelial cell adhesion molecule; NS, not significant; wt, wild-type.

these biomarkers and clinicopathological features and their impact on OS using Kaplan-Meier curves, the χ^2 test and Fisher's exact test. A significant association was observed between EGFRvIII expression (>5% positive tumour cells) and focal status of the tumour (P=0.04). However, there was no significant association between any other biomarker and clinicopathological features (data not shown). Next, the present study investigated the impact of expression of EGFRvIII on the OS of patients with HCC (Table III). The expression of EGFRvIII (at >5% of tumour cells stained) was associated with poor OS in patients with HCC (Tables III and SII; Fig. 2). In addition, patients whose tumours expressed cytoplasmic EGFRvIII had a significantly worse OS compared with those who were EGFRvIII-negative (63.66 vs. 28.70 months; P=0.002; Table SII). The results of univariate and multivariate analyses further confirmed the expression of EGFRvIII at >5 and 20% cut off values and cytoplasmic expression of EGFRvIII to be independent biomarkers of worse OS (Fig. 2; Table III).

Of the HER family, patients with an HER-2 immunostaining intensity of 1+ had a significantly improved OS compared with that of HER-2-negative patients (66.75 vs. 43.56 months; P=0.04; Fig. 2; Table III). The results of univariate and multivariate analysis confirmed that the presence of HER-2 with an immunostaining intensity of 1+ was associated with improved OS, with a HR of 0.514 (P=0.04) and 0.43 (P=0.01) (Tables III and SII).

Of the two putative liver CSC biomarkers, the cytoplasmic expression of CD44 was associated with significantly improved OS (100.20 vs. 55.92 months; HR, 0.20; P=0.003; Fig. 2; Tables III and SII). By contrast, the membranous expression of CD44 was associated with poor OS (37.09 vs. 55.92 months), with a HR of 2.12 and 2.15 in univariate and multivariate analysis, respectively (P=0.04; Table III). In addition, patients with nuclear EpCAM expression had a significantly reduced OS (25.67 vs. 53.52 months; P=0.05; Table SII). Of these patients, only 1 patient (2%) exhibited cytoplasmic staining of EpCAM that was present in >50% of tumour cells and was associated with significantly reduced OS (6.00 vs. 53.52 months; P=0.002; Table SII). However, this requires validation by examination of tumour specimens in a larger group of patients.

Association between co-expression of HER family members, EGFRvIII, CD44, EpCAM and Ki67 and OS in patients with HCC. To date, to the best of our knowledge, there is no comprehensive study of the co-expression and prognostic significance of all members of the HER family and other biomarkers, and their association with OS in patients with liver cancer. At a cut off value of >5% of tumour cells with positive staining, co-expression of EGFRvIII/wtEGFR was present in 14% of the cases examined and associated with a poor OS (21.00 vs. 59.47 months; P=0.004; Fig. 2; Table SII).

In addition, co-expression of EGFRvIII with CD44, EpCAM or KI67 was associated with shorter OS, with a HR of 4.03 (P=0.03), 2.68 (P=0.03) and 2.73 (P=0.02), respectively (Fig. 2; Table III). Furthermore, in the multivariate analysis, the co-expression of EGFRvIII/CD44 and EGFRvIII/Ki67 was also associated with a shorter OS (Table III).

Impact of various targeted agents on the proliferation of human LCCLs. Finally, the present study examined the expression levels of HER family members, CD44, IGF IR and c-MET in a panel of seven LCCLs established from patients at different stages of disease. Overall, the majority of these cancer cell lines expressed low levels of the HER family members, IGF IR and c-MET (Table IV; Fig. S1). By contrast, the expression levels of the CSC marker CD44 were high in four out of the seven LCCLs (Table IV; Fig. S1). Due

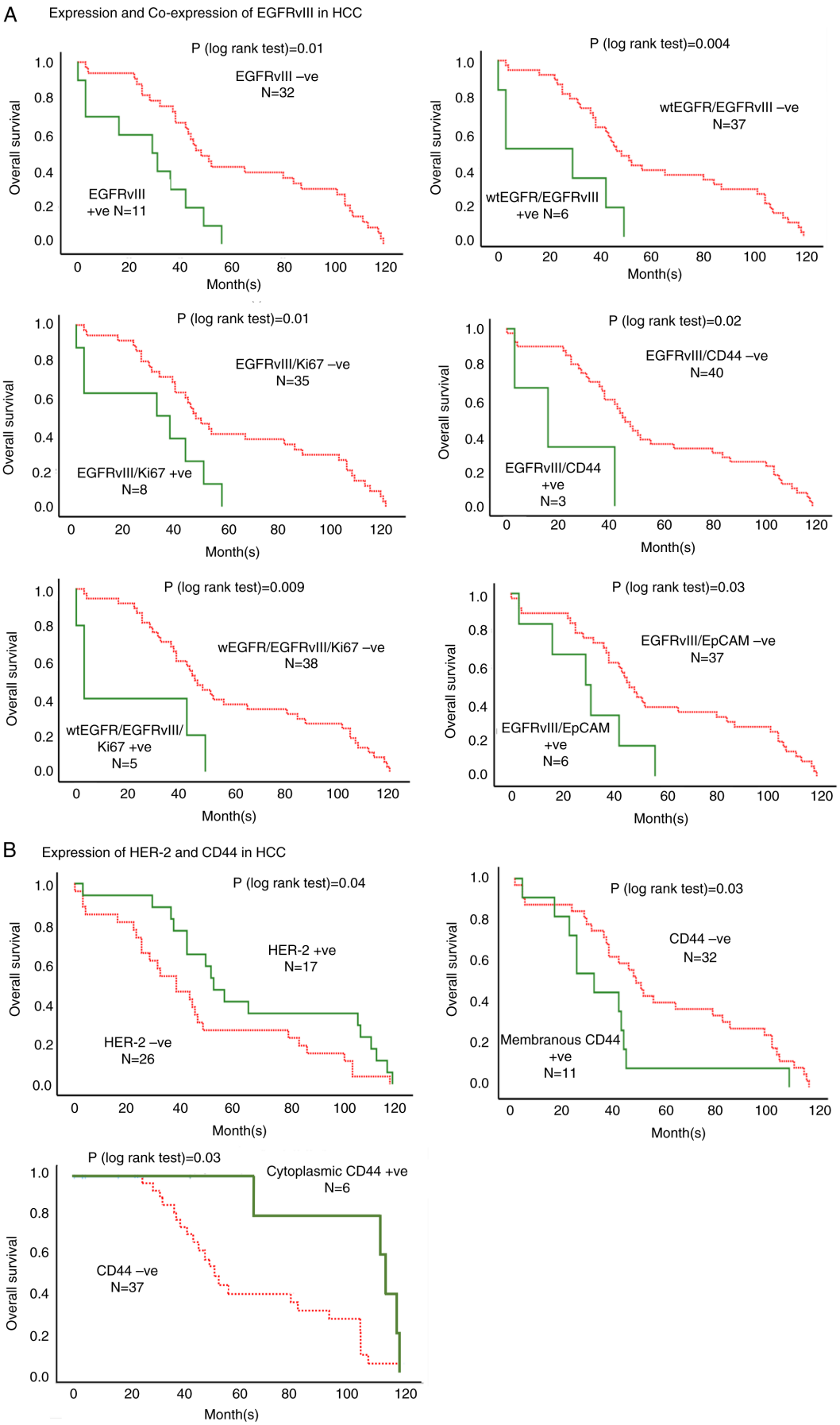

Figure 2. (A) Impact of the expression and co-expression of biomarker EGFRvIII on OS in patients with HCC. Kaplan-Meier survival curves for OS in patients with HCC. (B) Impact of the expression of biomarkers HER-2 and CD44 on OS in patients with HCC. Kaplan-Meier survival curves for OS in patients with HCC. EGFRvIII, type-III deletion mutant EGFR; EpCAM, epithelial cell adhesion molecule; HCC, hepatocellular carcinoma; OS, overall survival; wt, wild-type.

Table IV. Cell surface expression of HER family members, IGF IR, C-MET and CD44 in liver cancer cell lines determined by flow cytometry.

	Mean fluorescence intensity							
Cell lines	Untreated Sample	EGFR	HER-2	HER-3	HER-4	IGF IR	c-MET	CD44
HEPG-2 (HCC)	6.6±1.0	14.9±0.1	11.8±1.5	10.4±0.1	7.3±0.2	52.7±2.5	10.1±0.9	17.3±0.6
PLC/PRF/5 (hepatoma)	5.9±0.2	30.6±0.3	18.1±0.7	17.9±3.5	10.1±0.4	26.9±0.4	12.3±0.3	15.7±2.9
C3A (derivative of HepG2)	4.9±0.1	6.2±0.1	13.9±4.1	10.3±0.2	4.0±1.0	33.7±0.1	17.7±0.4	5.1±2.2
SNU 449 (HCC; hepatitis B positive)	4.2±0.1	13.4±3.6	15.4±2.5	7.7±0.2	4.5±0.1	16.1±4.0	22.1±0.1	204.3±3.8
SNU 475 (HCC; hepatitis B positive)	4.8±0.1	40.7±1.1	14.3±0.2	6.2±0.1	8.71±0.0	13.1±0.4	8.3±0.1	349.5±2.0
SNU 387 (HCC; hepatitis B positive)	4.9±0.1	10.6±0.1	6.9±0.1	5.4±0.1	5.6±0.1	9.5±0.0	9.5±0.0	251.9±0.2
SNU 423 (HCC; hepatitis B positive)	4.6±0.8	10.1±1.6	9.1±1.9	4.5±1.8	4.7±0.9	8.3±0.2	10.5±1.8	622.4±0.5
Other cell lines								
HN5 (head and neck cancer)	6.3	857.5±4.9	n/a	n/a	n/a	n/a	n/a	n/a
Skov-3 (ovarian cancer)	2.1	n/a	550.7±3.8	n/a	n/a	n/a	n/a	n/a
Caco2 (colorectal cancer)	4.6	n/a	n/a	n/a	n/a	n/a	n/a	126.1±7.0

The level of expression is reported as the mean fluorescence intensity alongside the standard deviation (n=2). HCC, hepatocellular carcinoma; IGF IR, insulin-like growth factor 1 receptor; n/a, not applicable as these cell lines were used as positive control cell lines.

Table V. Proliferation of human liver cancer cell lines, when cultured in medium containing 2 vs. 10% FBS, following treatment with various HER family-targeted agents.

	IC_{50} value (μM)							
	Gefitinib (reversible selective EGFR TKI)		Lapatinib (reversible dual EGFR/HER-2 TKI)		Sapitinib (reversible pan EGFR/HER-2/HER-3 TKI)		Afatinib (irreversible pan EGFR/HER-2/ HER-4 TKI)	
Cell line	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium
HEPG-2	>10	>10	>10	>10	>10	>10	>10	>10
PLC/PRF/5	>10	>10	2.43±1.20	>10	>10	>10	0.09±0.44	3.54±2.57
C3A	>10	>10	>10	>10	>10	>10	>10	>10
SNU 449	5.83±2.87	>10	>10	>10	>10	>10	>10	>10
SNU 475	>10	>10	>10	>10	>10	>10	7.82±4.59	>10
SNU 387	>10	0.12±0.42	>10	0.90±0.70	6.37±2.54	>10	>10	>10
SNU 423	3.35±2.18	>10	>10	>10	9.57±0.48	>10	6.29±1.68	>10

Each value is the mean ± SD of the IC_{50} values (n=3). TKI, tyrosine kinase inhibitor.

to the heterogenous nature of liver cancer, the present study subsequently investigated the effect of 12 agents targeting one or more members of the HER family, other growth factor receptors and cyclin-dependent kinases compared with FDA-approved regorafenib and sorafenib on the proliferation of LCCLs. Of the HER inhibitors targeting one or more members of the HER family, the irreversible pan-HER family blocker afatinib was the most effective drug by inhibiting the proliferation of three out of seven human LCCLs (Table V). This was more evident when tumour cells were cultured in a lower concentration of serum (2% FBS rather than 10% FBS). In addition, while treatment with the targeted agents crizotinib, NVP-AEW742 and LGK-974 inhibited the proliferation of some LCCLs, they were less effective than the two targeted drugs sorafenib and regorafenib, and doxorubicin, which is approved for the treatment of patients with HCC (Table VI).

Table VI. Proliferation of human liver cancer cell lines, when cultured in medium containing 2 vs. 10% FBS, following treatment with small molecule targeted agents and doxorubicin.

	IC_{50} value (μM)							
	Crizotinib (c-MET/ALK TKI)		NVP-AEW 742 (IGF IR TKI)		LGK-974 (Wnt inhibitor)		Doxorubicin	
Cell line	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium
HEPG-2	>10	8.82±0.44	>10	7.34±0.99	>10	>10	0.22±0.65	0.89±0.03
PLC/PRF/5	>10	7.99±0.94	>10	8.45±0.75	>10	9.95±2.69	1.20±2.77	0.01
C3A	>10	7.89±0.55	>10	8.27±0.63	>10	>10	>10	>10
SNU 449	>10	>10	2.74±2.33	>10	>10	>10	0.34±0.93	0.85±0.47
SNU 475	>10	>10	>10	>10	>10	>10	0.03±1.74	0.02
SNU 387	>10	9.74±0.14	4.81±1.91	>10	>10	9.89±1.34	0.60±1.87	0.16±0.14
SNU 423	>10	>10	>10	>10	>10	>10	0.01±0.84	0.01

Each value is the mean ± SD of the IC_{50} values (n=3). IGF IR, insulin-like growth factor 1 receptor; TKI, tyrosine kinase inhibitor.

Table VII. Proliferation of human liver cancer cell lines, when cultured in medium containing 2 vs. 10% FBS, following treatment with CDK inhibitors and Food and Drug Administration-approved sorafenib and regorafenib.

	IC_{50} value (μM)							
	Palbociclib (CDK4/6 inhibitor)		Dinaciclib (CDK1/2/5/9 inhibitor)		Sorafenib (RAF1/BRAF/ VEGFR1/2/4, PDGFR/KIT/ FLT3/FGFR1/RET inhibitor)		Regorafenib (VEGFR/VEGFR2/ KDR/VEGFR3/FLT4/ FGFR1/TEK/RAF1 inhibitor)	
Cell line	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium	2% FBS medium	10% FBS medium
HEPG-2	9.29±1.10	>10	5.09±3.75	3.21±2.12	8.10±1.88	7.39±2.05	8.26±1.57	6.22±1.45
PLC/PRF/5	0.22±0.93	8.44±0.44	0.02±0.98	0.95±1.01	0.37±0.47	3.86±1.11	0.37±0.52	3.40±0.05
C3A	>10	9.66±0.11	8.92±0.72	0.74±0.75	2.09±1.07	6.05±0.58	2.43±1.62	8.20±1.22
SNU 449	9.50±2.42	8.66±2.11	0.18±0.72	8.62±1.20	2.60±0.99	8.32±0.47	2.08±0.88	8.96±1.02
SNU 475	0.09±0.88	0.78±0.87	0.02±0.89	1.04±0.87	1.92±1.53	1.46±0.86	1.35±0.66	1.01±0.89
SNU 387	4.09±2.17	8.84±1.35	0.01±0.67	0.97±0.95	2.10±2.15	1.10±0.24	0.72±0.78	3.01±1.12
SNU 423	0.67±1.10	0.23±0.26	0.47±0.44	1.22±0.66	2.52±1.69	4.09±1.21	1.98±1.22	1.96±0.78

Each value is the mean ± SD of the IC_{50} values (n=3). FGFR1, fibroblast growth factor receptor 1; FLT, fms related receptor tyrosine kinase; KDR, kinase insert domain receptor; PDGFR, platelet derived growth factor receptor; TEK, TEK receptor tyrosine kinase.

Finally of the two CDK inhibitors, dinaciclib (CDK1/2/5/9 inhibitor) was the most effective targeted agent by inhibiting the proliferation of all LCCLs, being more effective than the CDK4/6 inhibitor palbocicilib, and sorafenib and regorafenib. Treatment with afatinib and dinacicilib inhibited the proliferation of human LCCLs (Table VII).

Discussion

HCC was the third most common cause of cancer-related deaths worldwide in 2020 (1). Although there are more effective therapeutic interventions for patients who are diagnosed at the early stage of cancer, the great majority of patients with HCC are diagnosed at the advanced stage of the disease and consequently have a poorer response to current treatments with both traditional and targeted cancer therapeutics (2,4,18). The difference between tumours from different patients with HCC (inter-tumour heterogeneity), and the presence of distinct tumour cell populations within the same tumour specimen (intra-tumour heterogeneity) and its microenvironment are some of the factors contributing to the primary and acquired tumour cell resistance to current therapies (2,4-5,7,9-10).

Therefore, in addition to preventative measures of avoiding the known risk factors such as hepatitis B virus or hepatitis C virus infections, to reduce the incidence and mortality of HCC, it is considered important to identify additional biomarkers of diagnostic, prognostic and predictive value in patients with HCC. It is also important to identify more specific therapeutic targets and to develop more effective therapeutic interventions.

EGFR and HER-2 are two of the most important receptors for targeted therapy in patients with a wide range of epithelial tumours, including colorectal, breast, lung, stomach, thyroid, and head and neck cancer, in combination with chemotherapy and radiotherapy (14-17). Despite the approval of several types of HER-targeted drugs, including naked mAbs, antibody-drug conjugates and small molecule TKIs targeting one or more members of the HER family, no such drugs have currently been approved for the treatment of patients with HCC (2,16-18). In some studies, the cross-talk between different members of the HER family and the cellular location of HER family members, such as membranous or nuclear EGFR, have been associated with tumour progression and poor response to treatment with HER inhibitors and other therapeutics (24-33). While some studies have investigated the expression of the individual members of the HER family in patients with HCC, to the best of our knowledge, there has been no comprehensive study of the co-expression, cellular location and prognostic significance of all members of the HER family and EGFRvIII, which is the most common deletion mutant type of EGFR at its extracellular domain, in patients with HCC. Therefore, to the best of our knowledge, the present study was the first to investigate the relative expression, cellular location and prognostic significance of all members of the HER family and EGFRvIII, as well as the two putative CSC biomarkers CD44 and EpCAM, and the cell proliferation biomarker Ki67 in patients with HCC (10,34,35). The present study investigated the expression levels and cellular location of wtEGFR, which transmits the mitogenic action of seven ligands belonging to the EGF family, including EGF, TGFα, amphiregulin, heparin-binding EGF, betacellulin, epiregulin and epigen, in patients with HCC. wtEGFR is recognized as the signalling hub for mediating the mitogenic action of its ligands via cross-talk with other growth factor signalling pathways. In particular, EGFR and its ligands have been implicated in wound repair and liver regeneration, and are also aberrantly expressed in the development of HCC (3,4-6). In the present study, at a cut off value of >5% of the tumour cells with staining, 35% of the cases were wtEGFR-positive. The cellular location of EGFR staining was membranous and cytoplasmic in 33 and 12% of cases, respectively, with 7% of the cases having an EGFR intensity of 2+. None of the cases exhibited nuclear staining of wtEGFR. Nuclear EGFR has been associated with poor prognosis and resistance to therapy in other cancer types, and may be a potential molecular target for anticancer strategies (36-38). At the same cut off value, 58% of the cases were HER-2-positive, and 14 and 40% of cases exhibited membranous expression of HER-2 and a staining intensity of 2+, respectively. Only HER-2 staining at an intensity of 1+ was associated with improved prognosis, and this warrants further investigation. While 19% of the cases were HER-4-positive, none of the cases were HER-3-positive in the present study.

Since the mid-1990s, membranous EGFR and HER-2 have been important targets for therapy with various types of mAb-based products (such as naked antibodies, antibody-drug conjugates, bispecific antibodies or chimeric antigen receptor T cells) in a wide range of epithelial tumours. However, the clinical benefit obtained may be modest in some patients, with the expression level, cellular location and mutational status of such receptors, and the heterogenous expression of HER family members being some of the contributing factors (14-17,39,40). In the present study, wtEGFR expression was accompanied by co-expression of HER-2 in 23% of the cases examined. It has been shown recently that treatment of patients even with low HER-2 (immunohistochemistry score of 2+/*in situ* hybridization-negative) metastatic breast cancer with the anti-HER-2 antibody-drug conjugate fam-trastuzumab deruxtecan-mxki resulted in improved OS in such patients, gaining FDA approval in August 2022 (41,42). Therefore, the present results support the need for investigation of the therapeutic potential of various types of mAb-based drugs and small molecule TKIs targeting the HER family members in patients with HCC whose tumours co-express these receptors.

In addition to the examination of co-expression of all members of the HER family, the present study investigated the relative expression and prognostic significance of EGFRvIII, which is the most common deletion mutant of EGFR containing deletions in exons 2-7 of EGFR. EGFRvIII contains a tumour specific, truncated extracellular domain incapable of ligand binding and is constitutively active (14-16,19). At the cut off value of >5% of the tumour cells with staining, 26% of the cases were EGFRvIII-positive. Notably, EGFRvIII expression alone at all cut off values of >5, 10 and 20% of cells with staining, and its co-expression with wtEGFR when the staining was present in >5% of the tumour cells were associated with poor prognosis, which was statistically significant in both univariate and multivariate analysis, with the exception of EGFRvIII staining at the cut off value of >10% of the tumour cells, which was statistically significant only in univariate analysis. EGFRvIII is currently an important target for therapeutic interventions in patients with glioblastoma with various types of mAb-based products, CAR-T cells, small molecule TKIs and EGFRvIII cancer vaccines (43-50). However, the clinical benefits of drugs targeting EGFRvIII have been modest. As cytoplasmic EGFRvIII will not be accessible to mAb-based drugs, the cellular location of EGFRvIII may also contribute to the poor response to therapy with such drugs (44-50). In the present study, EGFRvIII expression was mainly cytoplasmic, but its expression was associated with poor OS in patients with HCC. Therefore, further investigations of the co-expression, cellular location, prognostic significance and predictive value of EGFRvIII and other members of the HER family in a larger group of patients with HCC are warranted. The therapeutic potential of drugs targeting one or more members of the HER family when used in combination with other drugs in patients with HCC should also be evaluated (51).

In the past few decades, the heterogeneous nature of HCC has been associated not only with the presence of a diverse population of bulky tumour cells, but also with the presence of minor populations of CSCs in the tumour microenvironment. These are capable of self-renewal and differentiation

into different populations of tumour cells, leading to tumour growth, metastasis, tumour recurrence and resistance to therapies (52-55). As a result, the present study examined the expression of two putative CSC markers, CD44 and EpCAM, in patients with HCC (56-60). At the cut off value of >5% of tumour cells with staining, 40 and 33% of the cases were CD44-positive and EpCAM-positive, respectively. The cellular location of CD44 staining was membranous and cytoplasmic in 26 and 14%, respectively, of the cases examined. The membranous expression of CD44 was significantly associated with poor OS in patients with HCC in both univariate and multivariate analysis. By contrast, the cytoplasmic expression of CD44 was associated with improved OS (100.20 vs. 55.92 months; P=0.01). The results of a meta-analysis involving 14 studies and 2,225 cases showed that CD44 expression was associated with poor OS in patients with HCC (61). More recently, emodin, which is a natural product found in the roots of a number of plants and an anthraquinone derivate, has been shown to inhibit the proliferation of CD44-positive tumour cells. Further research should investigate whether the antitumour activity of emodin in HCC cells is accompanied by increased toxicity (60,61). While none of the patients in the present study exhibited nuclear staining of CD44, 7% of the cases exhibited nuclear expression of EpCAM, and the nuclear expression of EpCAM was associated with poor prognosis (25.67 vs. 53.52 months). At a cut off value of >5% of tumour cells with staining, not only the expression of EGFRvIII alone but also its co-expression with the two HCC CSC markers CD44 and EpCAM, the cell proliferation marker Ki67, wtEGFR, and wtEGFR/Ki67 were associated with poor OS in patients with HCC in the univariate analysis. The present results provide further support for the study of the expression pattern, prognostic significance and targeting of EGFRvIII in patients with HCC.

Finally, due to the heterogenous nature of HCC cells, the present study also investigated the relative expression of all members of the HER family, two other growth factor receptors (IGF IR and c-MET) and CD44 in a panel of human LCCLs. As increased proliferation is a hallmark of human cancers, the sensitivity of human LCCLs to treatment with agents targeting one or more members of the HER family, IGF IR, c-MET and Wnt, and treatment with two CDK inhibitors (palbocicilib and dinacicilib) was compared with the sensitivity to treatment with the two FDA-approved drugs sorafenib and regorafenib, and the cytotoxic drug doxorubicin. In general, the expression of HER family members in LCCLs was lower than that of EGFR and HER-2 in the positive control HN5 and SKOV3 cells, and four out of seven LCCLs exhibited upregulation of CD44 expression. As a result, of the HER inhibitors, the irreversible pan-HER family blocker afatinib was the most effective drug by inhibiting the proliferation of three out of seven human LCCLs, suggesting that not all HCCs depend on HER signalling for their proliferation. While treatment with crizotinib, NVP-AEW742 and LGK-974 inhibited the proliferation of some LCCLs, they were less effective than the two targeted drugs sorafenib and regorafenib, and doxorubicin, which is approved for the treatment of patients with HCC. Finally of the two CDK inhibitors, dinaciclib (CDK1/2/5/9) was the most effective targeted agent by inhibiting the proliferation of all LCCLs, being more effective than the CDK4/6 inhibitor palbocicilib and the FDA-approved sorafenib and regorafenib. Therefore, it would be interesting to investigate the therapeutic potential of drugs targeting EGFRvIII, CDKs and other members of the HER family in patients with HCC whose tumours are dependent on EGFRvIII and other members of the HER family for cancer growth.

In summary, to the best of our knowledge, the present study was the first to demonstrate that EGFRvIII expression occurred in patients with HCC. While the expression of EGFRvIII was mainly cytoplasmic, it was associated with poor survival when expressed alone or in combination with wtEGFR or CSC biomarkers. The present results highlighted the importance of EGFRvIII, which is a tumour-specific and constitutively active form of EGFR, as a biomarker of tumour progression. These results support targeting EGFRvIII by repurposing drugs specific for EGFRvIII and other members of the HER family when used in combination with CDK inhibitors and other FDA-approved drugs in patients with HCC.

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Availability of data and material

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HM conceived the original research idea for the project. OS performed the experiments and conducted data analysis. Both SAK and AMS co-supervised the project and helped with technical training and data analysis. IB and OS performed the scoring of immunohistochemistry staining. AD and SM were other collaborators on this project on the clinical study of EGFR expression in human cancers and made substantial contributions to acquisition of data and/or analysis and interpretation of data. OS wrote the manuscript. HM and SAK helped with final editing of the manuscript and confirm the authenticity of all the raw data. All authors have read and approved final version of the manuscript.

Ethics approval and consent to participate

The present retrospective study was approved by the NHS Research Ethics Committee (IRAS Project ID 252931; UK).

Due to the retrospective nature of the study, the requirement for patient consent was waived.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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