Clinical Impact of Intratumoral EpCAM-Positive Cancer Stem Cell Heterogeneity in Patients with Hepatocellular Carcinoma

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Abstract

Backgrounds & Aims: Intratumoural heterogeneity of hepatocellular carcinoma (HCC) is of increasing translational interest. Dismal prognosis is frequently linked to HCC harbouring cancer stem cell (CSC)-features, represented by EpCAM-expression. However, to what extent intratumoural distribution of CSC-features impacts on recurrence after curative resection remains unknown. Hence, we aimed to investigate the spatial heterogeneity of CSC-features and its impact on clinical outcome, identifying high-risk patients amenable to adjuvant treatment.

Methods: We designed a tissue microarray (TMA) from patients, who received liver resection between 2011 and 2017. Tumour specimens were sampled at multiple locations (n=3-8). EpCAM-positivity was assessed for intensity and proportion by applying a score dividing three groups: negative (E/-), heterogeneous-positive (E-/+), homogeneous-positive (E+/+). The groups were further analysed with respect to time-to-recurrence (TTR) and recurrence-free-survival (RFS).

Results: We included 341 tumour spots from 75 patients (77% male, median age 66 years, liver cirrhosis/fibrosis 74.6%). Risk factors were alcohol abuse in 23.9%, NASH 16.3%, HBV 14.1%, HCV 17.4% and others 28.3%, representing a typical Western cohort. E+/+ patients experienced a significantly shorter TTR and RFS compared to E-/- (and E-/) patients (TTR 5 vs. 19 months, p=0.017; RFS 5 vs. 14 vs. 18 months, p=0.016). Only homogeneous EpCAM-positivity correlated with higher AFP levels (>400 ng/ml, p=0.024).

Conclusions: Spatial heterogeneity of EpCAM-expression was markedly present. Only homogeneously positive EpCAM-expression correlated significantly with early recurrence, whereas heterogeneous EpCAM-expression was associated with clinical endpoints comparable to EpCAM-negativity. Similar to colorectal cancer, high or low risk features for recurrence could be decisive for adjuvant treatment.

Background

With approximately 90%, hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the 4th leading cause of cancer-related death worldwide\(^1\). HCC develops in 70–90% in patients with chronic liver disease, mainly liver cirrhosis due to chronic hepatitis B or C, alcohol abuse, non-alcoholic steatohepatitis (NASH) and rare causes such as hemochromatosis. Patients are a clinical highly heterogeneous group regarding the diversity of aetiologies and presence or absence of underlying liver disease. This is linked to an intertumoural molecular and histopathological heterogeneity. Rising evidence underlines an additional significance of intratumoural heterogeneity for treatment results. Friemel et al. demonstrated an intratumoural diversity in 87% of cases by applying morphological and immunohistochemical parameters in a small group of patients\(^2\). However, the clinical impact of tumour heterogeneity on tumour aggressiveness, early dissemination, and progression has not been thoroughly investigated so far.
HCC harbouring cancer stem cell (CSC)-features have a dismal prognosis, due to the higher invasiveness and greater potential in metastases-dissemination. As major breakthrough, Yamashita et al. identified EpCAM-positive cancer cells subpopulations in HCC with potential for self-renewal, de-differentiation, tumour-initiation, invasiveness, and the capacity to format distant metastases\textsuperscript{3, 4}.

To what degree the intensity and spatial distribution of intratumoural EpCAM-expression have an impact on local aggressiveness and metastases-dissemination remains unclear. High or low risk features for early metastases-dissemination, local aggressiveness, and recurrence could be a factor decisive of adjuvant treatment. So far, no adjuvant treatment has proven to be beneficial in HCC, despite high rates of recurrence of 50–70% within the first two years.

Therefore, we aimed to confirm spatial heterogeneity of CSC-features characterized by the intensity and proportion of EpCAM-positive cell-populations within different sections of HCC-nodules. We investigated the clinical impact of intratumoural heterogeneity on outcome, discriminating three pre-defined groups: EpCAM-negative (E-/−), heterogeneous-positive (E-/+), homogeneous-positive (E+/+). Finally, we correlated intratumoural EpCAM heterogeneity with serum AFP-levels, a surrogate marker for poor prognosis, and major clinical endpoints such as TTR and RFS.

**Methods**

**Patients:**

For this study, we screened the medical records of 987 patients with the diagnosis of HCC, who were treated at the University Medical Centre Hamburg-Eppendorf between July 2011 and October 2017. 75 patients underwent liver resection (LR) and sufficient tumour tissue was available for preparing a tissue micro-array (TMA). Patients were followed during the post-operative course and recurrence was diagnosed applying cross-sectional imaging, as recommended by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) guidelines.\textsuperscript{5, 6} In case of inconclusive contrast dynamics histological confirmation by biopsy was performed. Exclusion criteria were age < 18 years and any active or pre-existing concurrent malignancy. The study was approved by the Ethics Committee of the Hamburg Medical Association (approval number PV-3578) and written informed consent to the study protocol was obtained from all participants prior to inclusion in this study. Patients were not limited to any type or line of further treatment. Prior to specific TMA- and immunohistochemistry (IHC)-analysis, tissue-samples were reviewed by two independent pathologists from the Institute of Pathology of the University Medical Centre Hamburg-Eppendorf.

**Clinical characteristics:**

Baseline characteristics such as demographic and other clinical parameters (performance status, risk factors, underlying chronic liver disease, tumour stage according to the TNM and BCLC classification), laboratory data (AFP, bilirubin, albumin, and INR) and imaging were recorded prior LR. After LR, the pathological staging was incorporated into the analysis and surveillance of patients was performed with
regular contrast-enhanced cross-sectional imaging of the liver, clinical examination, and laboratory testing every three months according to guideline recommendations. All patients were followed up until recurrence of HCC or effective time of data analysis in July 2019.

Tissue microarray:

A TMA containing 341 tumour spots from the before mentioned 75 patients was constructed. Additional 90 liver tissue samples from patients with chronic liver disease were included as controls. Hematoxylin and eosin (H&E)-stained sections from all paraffin-embedded specimens were reviewed and tumour areas as well as adjacent liver tissue were marked on the slides. To generate a TMA mapping tumour heterogeneity, five 0.6-mm tissue cores were punched out from 3 to 8 different locations of the index area from 1 to 3 different paraffin-embedded tissue specimens, respectively, and transferred into a TMA format as previously described by Kononen.

Immunohistochemistry:

Freshly cut TMA sections were stained for EpCAM. High-temperature pretreatment of slides was done in a pressure cooker (DAKO buffer, pH 6.1, S1699) for 20 min. IHC was performed using a monoclonal antibody (1:10, clone VU-1D9, Novocastra, Newcastle, UK) to detect the membrane-bound positivity for EpCAM protein. The Envision system® (DAKO, Glostrup, Denmark) was used for visualization. Staining intensities and proportions of positive tumour cells were analysed for each tissue spot as proposed by the literature. A four-staged scale (0 = negative, 1 = weak, 2 = moderate and 3 = strong) was deducted for intensity, and (0 = negative, 1 < 10%, 2 = 10–50%, 3 > 50%, and 4 > 75% positivity) for proportion of positive tumour cells. Figure 1 displays representative examples for different staining results with respect to proportion and intensity of EpCAM-expression. Due to the size of the nodule, we yielded different number of spots per tumour. Finally, heterogeneity of EpCAM-expression (intensity and proportion) was assessed by a pre-defined score, discriminating three groups: EpCAM-negative (E-/­), heterogeneous-positive (E-/+), homogeneous-positive (E+/+). We considered both, proportion and intensity, of EpCAM-expression within one nodule as major criteria for the classification. In detail, a heterogeneous EpCAM profile (E-/+ is defined, if at least one spot is graded 0 and one spot at least 1 for intensity or proportion of EpCAM-expression.

Statistical Analysis:

All analysis was performed using R (version 3.6, R Foundation for Statistical Computing). Univariate comparisons of clinical data between pre-defined EpCAM-classification groups were performed using Fisher's Exact test for parametric data and Kruskal-Wallis-test for continuous data. Survival analyses and Cox-Regression modelling was performed with the survival package (version 2.38). Differences in survival rates at different time points were assessed by administrative censoring of the survival curves at the respective time point and performing a log-rank test implemented in the survdiff function.

Positive and negative prediction values, as well as sensitivity and specificity of a single spot were calculated by averaging over 1000 bootstrap samplings of single spots per sample. Classification
performance metrics for single spot and all spot prediction of tumour recurrence based on EpCAM intensity and proportion were calculated using the confusionMatrix function provided by the caret package (version 6.0.84). P-values below 0.05 were deemed statistically significant.

**Results**

Male patients were predominant [n = 58 (77.3%)]. The mean age was 66 years, ranging from 18 to 84 years. 41 (64.1%) of the cases were classified BCLC stage A. BCLC stage B [n = 18 (28.1%)] was less frequent, and a few cases with stage C [n = 5 (7.8%)] were included. The majority of patients (98.6%) were fully active or at least able to carry out work of a light or sedentary nature according to the Eastern Cooperative Oncology Group (ECOG 0 and 1). Chronic liver disease was common as underlying morbidity in our patients. Within our cohort, 40.3% of patients had liver cirrhosis (Child-Pugh A 88.5% and B 11.5%) and 25.4% of patients were treated without any histological signs of fibrosis. The rest of patients revealed a fibrosis (34.3%). Known aetiologies for HCC were equally distributed across the cohort with alcohol abuse [22 (24.2%)], chronic hepatitis C [16 (17.6%)] and B [13 (14.3%)], non-alcoholic steatohepatitis (NASH) [15 (16.5%)], and rare causes or no known underlying risk factor [25 (27.5%)]. More than two thirds of the patients (71.6%) underwent resection for a single lesion; only a minority presented 4 or more HCC-nodules (10.4%). Pathological reviewing classified 76% of patients T1 or T2, 99% exhibited N0, 97% L0, and 91% R0. 61% of patients revealed neither microscopic nor macroscopic vascular invasion. The majority (69%) of tumours were moderately differentiated (G2), 17% well differentiated (G1), and 14% poorly differentiated (G3). Overall, our patients represented a typical cohort undergoing curative resection in the Western World. Clinical baseline and general histopathological tumour characteristics are summarized in Table 1.
Table 1
Clinical variables for the overall cohort. Aetiology variable may include multiples risk factors.

| Baseline Characteristics | Overall n = 75 (%) |
|--------------------------|-------------------|
| Gender                   |                   |
| m                        | 58 (77.33)        |
| f                        | 17 (22.67)        |
| Age at surgical procedure (years) |                   |
| Range                    | 18-84             |
| Median (n)               | 66 (75)           |
| ECOG (n = 70)            |                   |
| 0                        | 39 (55.71)        |
| 1                        | 30 (42.86)        |
| 2                        | 1 (1.43)          |
| BCLC (n = 74)            |                   |
| A                        | 41 (64.06)        |
| B                        | 18 (28.12)        |
| C                        | 5 (7.81)          |
| Histological Stage of Liver (n = 67) |                   |
| No Fibrosis              | 17 (25.37)        |
| Fibrosis                 | 23 (34.33)        |
| Cirrhosis                | 27 (40.30)        |
| Child-Pugh Stage (n = 26) |                   |
| A                        | 23 (88.46)        |
| B                        | 3 (11.54)         |
| Etiology                 |                   |
| Alcohol                  | 22 (24.18)        |
| Hepatitis B              | 13 (14.29)        |
| Hepatitis C              | 16 (17.58)        |
| NASH                     | 15 (16.48)        |
| Number of HCC-lesions (n = 67) |   |   |
|-------------------------------|---|---|
| 1                     | 48 (71.64) |   |
| 2                     | 6 (8.96) |   |
| 3                     | 6 (8.96) |   |
| >=4                   | 7 (10.45) |   |
| T stage               |   |   |
| T1                   | 28 (40.00) |   |
| T2                   | 25 (35.71) |   |
| T3                   | 17 (24.29) |   |
| N stage               |   |   |
| N0                   | 69 (98.57) |   |
| N1                   | 1 (1.43) |   |
| L stage               |   |   |
| L0                   | 69 (97.18) |   |
| L1                   | 2 (2.82) |   |
| V stage               |   |   |
| V0                   | 43 (61.43) |   |
| V1                   | 27 (38.57) |   |
| R stage               |   |   |
| R0                   | 64 (91.43) |   |
| R1                   | 6 (8.57) |   |
| G stage               |   |   |
| G1                   | 12 (16.91) |   |
| G2                   | 49 (69.01) |   |
| G3                   | 10 (14.08) |   |

As indicated, the primary aim was to confirm the presence of spatial heterogeneity of CSC-features, measured by intensity and proportion of EpCAM-positivity within the same tumour nodule. 32 patients
(42.7%) did not exhibit any EpCAM-expression (E-/-), whereas 7 patients (9.3%) stood out with EpCAM-expression throughout all tumour spots (homogeneous positivity, E+/+). 36 patients (48%) contained tumour spots with and without EpCAM-expression of diverse quality, confirming the presence of spatial heterogeneity of CSC-features in nearly half of patients with HCC. Figure 2 illustrates the results of EpCAM-staining in intensity and proportion in all 341 tumour spots with respect to EpCAM-classification score [negative (E-/-), heterogeneous-positive (E-/+), homogeneous-positive (E+/+)] and in correlation with baseline features. Primarily, Fig. 2 visualizes the fact, that E+/+ patients separate from E+-/ and E-/- patients in the context of tumour aggressiveness (serum AFP-levels (</≥ 400 ng/mL) and number of HCC nodules) and the major clinical outcome of recurrence within 2 years of resection. Remarkably, E+-/ and E-/- demonstrate similar clinical behaviour, serum AFP-levels, and number of HCC nodules.

Based upon the EpCAM-score [negative (E-/-), heterogeneous-positive (E-/+), homogeneous-positive (E+/+)], we were interested in the outcome of the patients. First, we checked for group comparability with respect to the baseline characteristics (Table 2). We did not find any significant differences in demographic data, performance status, risk factors, presence or absence of underlying chronic liver disease, liver function, or staging according to the BCLC classification. Likewise, there were no significant differences between the three groups with respect to the L-, V-, N-, R- or G-stage. Overall, we did not identify any confounders between the three groups for our final outcome analysis.
Table 2

**Cohort split into EpCAM classification groups.** Group differences assessed by Fisher’s Exact test for categorical variables and Kruskal-Wallis-test for numerical variables.

| Baseline Characteristics | Overall n = 75 (%) |
|--------------------------|-------------------|
| Gender                   |                   |
| m                        | 58 (77.33)        |
| f                        | 17 (22.67)        |
| Age at surgical procedure (years) |       |
| Range                    | 18–84             |
| Median (n)               | 66 (75)           |
| ECOG (n = 70)            |                   |
| 0                        | 39 (55.71)        |
| 1                        | 30 (42.86)        |
| 2                        | 1 (1.43)          |
| BCLC (n = 74)            |                   |
| A                        | 41 (64.06)        |
| B                        | 18 (28.12)        |
| C                        | 5 (7.81)          |
| Histological Stage of Liver (n = 67) |            |
| No Fibrosis              | 17 (25.37)        |
| Fibrosis                 | 23 (34.33)        |
| Cirrhosis                | 27 (40.30)        |
| Child-Pugh Stage (n = 26) |                   |
| A                        | 23 (88.46)        |
| B                        | 3 (11.54)         |
| Etiology                 |                   |
| Alcohol                  | 22 (24.18)        |
| Hepatitis B              | 13 (14.29)        |
| Hepatitis C              | 16 (17.58)        |
| NASH                     | 15 (16.48)        |
| Baseline Characteristics | Overall n = 75 (%) |
|--------------------------|------------------|
| Other                    | 10 (10.99)       |
| w/o known risk factor    | 15 (16.48)       |
| Number of HCC-lesions (n = 67) |           |
| 1                        | 48 (71.64)       |
| 2                        | 6 (8.96)         |
| 3                        | 6 (8.96)         |
| >=4                      | 7 (10.45)        |
| T stage                  |                  |
| T1                       | 28 (40.00)       |
| T2                       | 25 (35.71)       |
| T3                       | 17 (24.29)       |
| N stage                  |                  |
| N0                       | 69 (98.57)       |
| N1                       | 1 (1.43)         |
| L stage                  |                  |
| L0                       | 69 (97.18)       |
| L1                       | 2 (2.82)         |
| V stage                  |                  |
| V0                       | 43 (61.43)       |
| V1                       | 27 (38.57)       |
| R stage                  |                  |
| R0                       | 64 (91.43)       |
| R1                       | 6 (8.57)         |
| G stage                  |                  |
| G1                       | 12 (16.91)       |
| G2                       | 49 (69.01)       |
| G3                       | 10 (14.08)       |
To test for the clinical impact of EpCAM expression in HCC, we determined the TTR as well as RFS for early recurrence (i.e. within 24 months). Recurrence within the first 24 months after curative resection is considered more likely to be true recurrence instead of de novo tumours. As highlighted in Fig. 3, TTR is significantly shorter with 5 months in patients with E+/+ compared to patients with E+/- with 19 months (p = 0.017). The median TTR was not reached for patients scored E-/-.

The hazard ratio for recurrence between E+/+ and E-/- was 3.9 (95% CI, 1.36–11.2, p = 0.011). Results for RFS, depicted in Fig. 4, were also significantly different with 5 months for E+/+ compared to 14 months for E-/+ (p = 0.016).

The hazard ratio was 3.6 (95% CI, 1.4–9.1, p = 0.009). Interestingly, in both endpoints, TTR and RFS, we found similar outcome in E+/- and E-/- groups (Figs. 3 and 4). Recurrence-free survival rates at 24 months were 52% and 35% for the E-/- and E+/- group (p = n. s.), respectively. No patient in the E+/+ group remained without recurrence or death in 24 months after resection. In summary, we demonstrated significantly worse prognosis for patients with homogeneously positive EpCAM expression, and also showed that EpCAM-heterogeneous-positivity and EpCAM-negativity exhibited similar outcome, underscoring the clinical impact for thorough assessment of spatial EpCAM-expression in HCC nodules.

Further, we were interested in surrogate parameters for tumour aggressiveness in homogeneous EpCAM-positive HCC. As surrogate for local aggressiveness, tumour-initiation, and invasiveness, we assessed AFP serum levels prior to resection, and the number of satellite lesions. AFP levels were significantly higher in the E+/+ group compared to the other groups. In detail, 57% of patients with E+/+ presented AFP levels > 400 ng/mL compared to 13% in E+/- and 11% in E-/- (p = 0.024). Remarkably, heterogeneous EpCAM-expression was linked to similar low AFP-levels as in EpCAM-negative patients. Correspondingly, patients with E-/- and E+/- status harboured significantly less satellite tumour lesions at time of resection compared to the E+/+ group (p = 0.026) (Table 3.)
### Table 3
Surrogate markers for local aggressiveness, de-differentiation, tumour-initiation, and invasiveness in patients with HCC, considering EpCAM classification groups. Group differences assessed by Fisher’s Exact test for categorical variables and Kruskal-Wallis-test for numerical variables.

| Baseline Characteristics | EpCAM-negative n = 32 (42.7%) | EpCAM-positive heterogeneous n = 36 (48.0%) | EpCAM-positive homogeneous n = 7 (9.3%) | p-value |
|--------------------------|--------------------------------|---------------------------------------------|----------------------------------------|---------|
| Gender                   |                                |                                             |                                        |         |
| m                        | 25 (78.12)                     | 28 (77.78)                                 | 5 (71.43)                              | 0.851   |
| f                        | 7 (21.88)                      | 8 (22.22)                                  | 2 (28.57)                              |         |
| Age at surgical procedure (years) |                        |                                             |                                        |         |
| range                    | 38–84                          | 18–81                                       | 58–76                                  | 0.359   |
| median (n)               | 68.5 (32)                      | 66 (36)                                     | 63 (7)                                 |         |
| ECOG                     |                                |                                             |                                        |         |
| 0                        | 17 (56.67)                     | 20 (58.82)                                 | 2 (33.33)                              | 0.569   |
| 1                        | 12 (40.00)                     | 14 (41.18)                                 | 4 (66.67)                              |         |
| 2                        | 1 (3.33)                       | 0 (0)                                       | 0 (0)                                  |         |
| BCLC                     |                                |                                             |                                        |         |
| A                        | 20 (71.43)                     | 19 (63.33)                                 | 2 (33.33)                              | 0.103   |
| B                        | 8 (28.57)                      | 7 (23.33)                                  | 3 (50.00)                              |         |
| C                        | 0 (0)                          | 4 (13.33)                                  | 1 (16.67)                              |         |
| Histological Stage of Liver |                                      |                                             |                                        |         |
| No Fibrosis              | 7 (24.14)                      | 7 (22.58)                                  | 3 (42.86)                              | 0.818   |
| Fibrosis                 | 11 (37.93)                     | 10 (32.26)                                 | 2 (28.57)                              |         |
| Cirrhosis                | 11 (37.93)                     | 14 (45.16)                                 | 2 (28.57)                              |         |
| Child Pugh stage         |                                |                                             |                                        |         |
| A                        | 11 (100)                       | 11 (84.62)                                 | 1 (50.00)                              | 0.112   |
| B                        | 0 (0)                          | 2 (15.38)                                  | 1 (50.00)                              |         |
| Etiology                 |                                |                                             |                                        |         |
| Alcohol                  | 9 (22.50)                      | 11 (26.83)                                 | 2 (20.00)                              | 0.655   |
In summary, homogeneous distribution of EpCAM-expression was significantly indicative for higher local aggressiveness and earlier tumour dissemination compared to heterogeneous EpCAM-positivity. EpCAM-
negative tumour nodules demonstrated similar low aggressive tumour features like EpCAM-
heterogeneous tumour nodules.

Since only homogeneous EpCAM-positivity was associated with a more aggressive tumour behaviour
and subsequent poorer outcome, we were interested in the predictive value of a single biopsy towards the
overall EpCAM-score (E+/+, E+-/ or E-/). Therefore, we assessed the positive and negative prediction
values, as well as sensitivity and specificity of the EpCAM expression pattern of a single spot. We
discovered that the sensitivity for a single spot was 100%, the specificity was 87%, the negative predictive
value was 100%, but the positive predictive value was only 45%. Moreover, the positive predictive value of
a single spot for early recurrence within 24 months after resection was 55%, the negative predictive value
was 57%, the sensitivity was 25%, and the specificity was 83%.

Discussion

The phenotypic and histopathological characterization of malignancies is increasingly applied to tailor
personalized medicine in regards to the management of treatment decision-making. Adjuvant treatment
in patients with HCC is still not established because positive phase 3 clinical trials are lacking, probably
due to an insufficient patient selection in recent studies. It is believed that selected patients could benefit
from approved treatment modalities, e.g. transarterial chemoembolization (TACE), tyrosine kinase
inhibitors, immune checkpoint inhibitors, in an adjuvant setting under consideration of stringent selection
criteria. Similar to colorectal cancer, high or low risk features for early metastases-dissemination and
local aggressiveness could be key for adjuvant treatment after curative-intended resection. To this
respect, CSC-features, in particular EpCAM-expression, are therefore a highly attractive surrogate marker
in HCC. However, to what extent intratumoural heterogeneity of EpCAM expression is present in HCC
nodules and how it might affect clinical outcome of patients has not yet been evaluated in a large
enough patient cohort. The proportion of intratumoural CSC-features as well as the intensity of
expression-levels in each HCC nodule remains uncertain, and could provide an ideal marker for stringent
patient selection in an adjuvant treatment trial setting.

To our knowledge, this is the largest cohort of HCC patients to have been investigated precisely in terms
of presence or absence of intratumoural CSC heterogeneity. Herein, we could verify that CSC-features are
substantially heterogeneously distributed within HCC nodules. Homogeneous EpCAM staining was
present in only 1 out of 10 patients whereas EpCAM heterogeneity was found in every other patient. We
showed, that homogeneous distribution of CSC-features was leading to earlier dissemination, recurrence
and/or death after curative-intended resection with 5 months RFS for E+/+ patients compared to 14
months for E-/+. Based on our findings, we postulate that only homogeneous distribution of CSC-features
lead to more aggressive tumours, alongside with a higher risk of satellite nodules (i.e. local metastases).

Moreover, homogeneous distribution of CSC features was significantly associated with higher serum
AFP-levels (p = 0.048) and the number of intrahepatic satellite HCC lesions (p = 0.026) compared to
heterogeneous EpCAM expression. Again: homogeneous presence of CSC features was associated with
local aggressiveness and consecutively lead to shorter TTR and RFS. Of note, all E+/- and E-/- cases had similar clinical outcomes, suggesting the same biological behaviour in HCC with none or low expression pattern of CSC features.

Hepatic stem and progenitor cells can be the source of tumour initiation and CSC can drive hepatocarcinogenesis\(^\text{11,12}\). We here highlight, that only a full-scale CSC milieu within HCC nodules was of clinical significance in terms of prognosis. A subpopulation of cells with CSC features is responsible for sustainment of many different solid tumours\(^\text{13}\). However, a minor CSC cluster or single CSC appear not to be sufficient enough to influence tumour proliferation and metastases-dissemination.

Whether CSC features cause HCC aggressiveness, has to be further studied by functional experiments.

Substantial EpCAM expression is believed to be the result of a distinct underlying molecular and mutational profile as introduced by molecular classifications\(^\text{14}\). Herein, HCC can be categorized in a proliferation and non-proliferation class\(^\text{14}\). EpCAM positivity and abundant CSC features are associated with the G1/S2/iCluster 1, termed the progenitor group. This group is characterized by \textit{RPS6KA3}, \textit{TP53}, and \textit{AXIN1} mutations as well as IGF1R, AKT/mTOR signaling\(^\text{14}\). Thus, it will be crucial to identify the underlying mutational profile, eventually leading to HCC with homogeneous or heterogeneous CSC distribution. WNT-\(\beta\)-catenin activation via transcription factor TCF-4 is also associated with transcriptional activation of EpCAM expression\(^\text{15}\). A Japanese study demonstrated in two human HCC samples inactivating \textit{TP53} mutations (c.844C \(\rightarrow\) T; c.767C \(\rightarrow\) T) in EpCAM positive HCC\(^\text{12}\). In HCC, activating \textit{CTNNB1} mutations and inactivating \textit{TP53} mutations are major oncogenic events with frequencies of up to 37\% and 24\%, respectively, and could be in part reasonable drivers in homogeneous distribution in EpCAM expression\(^\text{16}\). Due to methodological limitations of the present study, analysis of the mutational background was not within the scope of this work, but upcoming studies will need to address the molecular and genetic impact on homogeneous or heterogeneous CSC patterns. Longitudinal sampling, focusing on clonal evolution, might also help clarifying this issue. Ultimately, it will be necessary to study CSC heterogeneity within a large patient cohort at a clonal level through single cell analysis. Nevertheless, with our comprehensive data we demonstrate that clinical parameters, mainly risk factors and presence of underlying chronic liver disease, are not involved in determining HCC with a homogeneous or heterogeneous CSC expression pattern.

We believe that accurate histopathological investigation of resected HCC lesions harbour important information to stratify patient management. With respect to intensity and proportion of CSC features, a simple EpCAM score could estimate the risk of recurrence. Subsequently, the score could be used to establish a clinical trial, which investigates adjuvant treatment options. Herein, patients could be stratified by the E+/+ status versus E+/- (and/or E-/-) status, followed by an adjuvant treatment.

Several trials in HCC, predominantly focusing on combinatory treatments with checkpoint inhibition, are currently on-going. It is well known that PD-1 or PD-L1 expression within the tumour lesion and microenvironment in general are not sufficient surrogate markers for treatment response in patients
receiving checkpoint inhibition in HCC\textsuperscript{14}. Recently, several study groups emphasized that mesenchymal stem cells suppress inflammation and inhibit the immune response by the PD-1/PD-L1 axis\textsuperscript{17,18}. To our knowledge there is only minor or no clinical evidence in large HCC cohorts to support the efficacy of immune checkpoint inhibitors in CSC-predominant subtypes of HCC. It could be elucidating to investigate the PD-1 and PD-L1 status in HCC nodules harbouring homogeneous CSC-distribution and its response to checkpoint inhibitors. Eventually, patients under mono-therapy of immune checkpoint inhibition need to be co-tested for EpCAM, PD-1, and PD-L1 expression in order to obtain the predictive value towards treatment response.

Our data set underlines again the presence and impact of intratumoural heterogeneity in HCC. We already know from other gastrointestinal cancer types that intratumoural heterogeneity can play a major role for treatment decision-making, e.g. HER2/neu expression in gastric cancer\textsuperscript{19}. In their study, sampling procedures contained a significant risk to generate a false-negative Her2/neu status. 25\% of patients with gastric cancer and Her2/neu overexpression have been neglected due to sampling error, leading to withholding of effective treatment with trastuzumab. Additionally, a minor false-positive rate of 6\% has been reported\textsuperscript{19}.

We addressed the issue of representative sampling, taking into account the EpCAM expression patterns of a single spot compared to all spots of one HCC nodule and its significance towards the EpCAM score. We discovered that every other patient with a positive EpCAM result harbours in fact heterogeneous EpCAM expression, and therefore has the same prognosis of a patient with an EpCAM negative HCC nodule. In conclusion, one single biopsy does not seem to be sufficient to predict patient outcome, regarding SCS-features and its impact on recurrence. The indication for performing biopsies in patients with late stage disease will be increasing in the future. Our results were generated from patients with early tumour stages. Thus, our conclusions cannot be transferred to patients with advanced tumour stages. However, we believe the subject of sampling error has to be addressed in patients with later stages, because cancers with CSC features are more resistant to systemic chemotherapy\textsuperscript{20}. The results of the present study and its impact on clinical outcome suggest that more than one biopsy is required to minimize the risk of false negative or positive predictive histopathological staining.

\textbf{Conclusion}

CSC intratumoural heterogeneity is present in HCC nodules as demonstrated in this large cohort. Only homogeneous distribution of EpCAM staining was associated with more tumour aggressiveness and metastases-initiation as well as worse outcome, indicated by shorter TTR and RFS. Tumour nodules with heterogeneous distribution of EpCAM staining have remarkably similar characteristics as tumour nodules without any EpCAM-positive cells. Especially in times of increasing significance of tumour biopsies in late stage disease our study finally underlines the risk of sampling error for clinical decision-making.

\textbf{Abbreviations}
AASLD American Association for the Study of Liver Diseases
AFP Alpha-Fetoprotein
AKT/mTOR Protein Kinase B/mechanistic Target of Rapamycin
AXIN1 Axis Inhibition Protein 1
BCLC Barcelona Clinic Liver Cancer Classification
CSC Cancer Stem Cell
CTNNB1 Catenin Beta 1
EASL European Association for the Study of the Liver
ECOG Eastern Cooperative Oncology Group
EpCAM Epithelial cell adhesion molecule
H&E Hematoxylin and Eosin
HBV Hepatitis B Virus
HCC Hepatocellular Carcinoma
HCV Hepatitis C Virus
HER2/neu Human Epidermal Growth Factor Receptor 2
IGF1R Insulin-Like Growth Factor 1
IHC Immunohistochemistry
INR International Normalized Ratio
LR Liver resection
NASH Non-alcoholic Steatohepatitis
PD-1 Programmed Death-1
PD-L1 Programmed Death Ligand-1
RFS Recurrence-Free-Survival
RPS6KA3 Ribosomal Protein S6 Kinase A3
TACE Transarterial Chemoembolization
TCF-4 Transcription factor 4
TMA Tissue Microarray
TNM TNM Classification of Malignant Tumours (T, tumour; N, lymph nodes; M, metastases)
TP53 Tumour suppressor protein 53
TTR Time-To-Recurrence
WNT Wingless-Type MMTV Integration Site Family

Declarations

Ethics approval and patient consent:

The study was approved by the Ethics Committee of the Hamburg Medical Association (approval number PV-3578) and written informed consent to the study protocol was obtained from all participants prior to inclusion in this study.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

KS, JK, JvF and HW designed the study. KS, JvF, TWF, CS and HW obtained patient data. JK, GS, TK and SAW designed the TMA and conducted the staining. KS, JK and JG performed the readout of the stained TMA. CJ and HI obtained radiological follow-up data. AH, JL and LF obtained tissue specimens. HW and AWL supervised the study. CC performed all statistical analyses. KS, JvF and JK wrote the manuscript. All authors have read and approved the manuscript.

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Authors’ information

JK has undergone specific training for gastrointestinal and hepatobiliary pathology for 12 months by GS and TK.

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Figures
Figure 1

Evaluation of EpCAM Intensity and Proportion. Proportion scoring (0=negative, 1<10%, 2=10-50%, 3>50%, and 4>75%), intensity scoring (0=negative, 1=weak, 2=moderate and 3=strong). Proportion/Intensity:
Panel A 0/0, B 2/1, C 3/2, D 4/1, E 4/2, F 4/3.

Figure 2

Heterogeneity of EpCAM-expression in HCC. Results of EpCAM-expression per TMA spot and patient are annotated by clinical metadata. Bottom half of the map visualises the block-wise, categorical EpCAM-
Expression intensity and proportion. Map cells are split vertically to indicate different levels for intensity/proportion.

Figure 3

Correlation of time-to-recurrence with EpCAM classification groups. A: Kaplan Meier analysis showing significantly earlier recurrence within 24 months after resection of HCC with homogeneous EpCAM expression, when compared to heterogeneous EpCAM-expressing and EpCAM-negative HCC. B. Cox-Regression model including hazard ratios showing higher risk for recurrence within 24 months of HCC with homogeneous EpCAM expression.
Figure 4

Correlation of recurrence-free survival with EpCAM classification groups. A: Kaplan Meier analysis showing significantly reduced recurrence-free survival of patients with HCC with homogeneous EpCAM expression, when compared to heterogeneous EpCAM-expressing and EpCAM-negative HCC. B. Cox-Regression model including hazard ratios showing higher risk for reduced recurrence-free survival of HCC with homogeneous EpCAM expression.