Whole exome sequencing identifies clinically relevant mutational signatures in resected hepatocellular carcinoma

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Abstract

Background & Aims: Most patients develop recurrent disease despite curative surgery for hepatocellular carcinoma (HCC). No standard adjuvant therapy is available and molecular predictors of outcome are poorly understood.

Methods: We conducted a multicentre pilot study on patients with localized HCC following surgical resection. Patients received up to 6 months of oral gefitinib as adjuvant therapy. Clinical end points included recurrence-free survival (RFS) and overall survival (OS), and exploratory analyses were conducted from whole exome sequencing data.

Results: A total of 65 patients were screened for the study, of which 40 were eligible. The median age was 63 years (range, 44-80). Most patients (80%) were positive for hepatitis B or C. With a median follow-up of 4.5 years, the median RFS was 24 months. Median OS was not reached. High Child-Pugh score and advanced T-stage (3-4) were independent predictors for both OS and RFS. Mutational signatures for exposure to aristolochic acid (AA), as characterized by a majority of T:A > A:T mutations, were observed in 18 cases (55%). HCC without AA mutagenesis was associated with early recurrences or death within 2 years of surgery (P = .037). HCC with high T > A mutations was associated with better OS (HR 0.29, 95% CI 0.09-0.90, P = .033). Conversely, HCC with high C > T mutations was associated with worse OS (HR 4.55, 95% CI 1.20-17.31, P = .026).

Conclusions: Mutational signatures may carry prognostic significance in HCC following curative resection. Patient outcomes with gefitinib as adjuvant therapy after...
Hepatocellular carcinoma (HCC) is a global health problem and a leading cause of cancer-related deaths worldwide. In particular, the poor prognosis of this disease encompasses the group of patients with localized disease at the time of diagnosis, with over half of them at risk of developing recurrent or metastatic disease despite curative liver resection and/or transplantation. Attempts to improve survival rates with adjuvant therapy following local therapy have thus far been disappointing, highlighting the need for urgent research in this area of unmet clinical need. ClinicalTrials.gov number, NCT00282100.

**KEYWORDS**
adjuvant therapy, aristolochic acid, mutational signatures, prognostic biomarker, tyrosine kinase inhibitor

Genomic footprints of exposure to aristolochic acids (AA) found in plants have been discovered in liver cancers across several parts of Asia. In a well-annotated cohort of patients with liver cancer treated within a prospective clinical trial, signatures of AA exposure were observed in over half the cases, confirming it as a major healthcare problem. Interestingly, the identification of AA signatures was correlated with better survival outcomes, supporting their potential relevance in the clinic.

**Key points**

Genomic footprints of exposure to aristolochic acids (AA) found in plants have been discovered in liver cancers across several parts of Asia. In a well-annotated cohort of patients with liver cancer treated within a prospective clinical trial, signatures of AA exposure were observed in over half the cases, confirming it as a major healthcare problem. Interestingly, the identification of AA signatures was correlated with better survival outcomes, supporting their potential relevance in the clinic.
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TABLE 1 Characteristics of the patients included in the study

| Characteristic          | n (%) |
|-------------------------|-------|
| Total                   | 40 (100) |
| Sex                     |       |
| Male                    | 36 (90) |
| Female                  | 4 (10) |
| Age at diagnosis (y)    |       |
| ≥65                     | 20 (50) |
| <65                     | 20 (50) |
| HBV status              |       |
| Positive                | 30 (75) |
| Negative                | 10 (25) |
| HCV status              |       |
| Positive                | 5 (12.5) |
| Negative                | 35 (87.5) |
| Cirrhosis               |       |
| Present                 | 20 (50) |
| Absent                  | 20 (50) |
| Child-Pugh score        |       |
| 5                       | 21 (52.5) |
| 6                       | 14 (35) |
| 7                       | 4 (10) |
| 8                       | 1 (2.5) |
| Serum AFP levels (µg/L) |       |
| ≤50                     | 36 (90) |
| >50                     | 4 (10) |
| T-stage                 |       |
| 1                       | 16 (40) |
| 2                       | 11 (27.5) |
| 3a                      | 5 (12.5) |
| 3b                      | 5 (12.5) |
| 4                       | 3 (7.5) |
| Multifocal              |       |
| Yes                     | 7 (17.5) |
| No                      | 33 (82.5) |
| Vascular invasion       |       |
| Present                 | 17 (42.5) |
| Absent                  | 23 (57.5) |
| Tumour size >5 cm       |       |
| Yes                     | 19 (47.5) |
| No                      | 21 (52.5) |
| Resection margins       |       |
| Positive                | 2 (5) |
| Negative                | 38 (95) |
| Early recurrence        |       |
| Yes                     | 20 (50) |
| No                      | 20 (50) |

based on the proportion of non-synonymous variants per coding megabase. Mutational signature assignment was performed with mSigAct v0.13. Biologically significant copy-number changes were identified with GISTIC 2.0 and copy-number segmentations were processed with TitanCNA v1.17.1. GenVisR was used to visualize the landscape of mutational alterations in this cohort.

2.5 Statistical analysis

Receiver operating curve (ROC) analysis via the method of DeLong et al was used to derive the optimal cut-off values for continuous parameters as univariable predictors of OS. Comparisons of the frequencies of categorical variables were performed using Fisher’s exact tests. Continuous variables and their associations with categorical variables were evaluated by Mann-Whitney U tests (non-normally distributed) or by t tests (normally distributed). Kaplan-Meier
analyses were conducted to identify statistically significant univariate predictors of survival, and represented by hazard ratios (HR) and 95% confidence intervals (95% CI). Multivariate Cox regression model via a stepwise procedure was employed to determine the independence of significant factors identified on univariable analysis. All statistical analyses were conducted assuming a two-sided test with significance level of 0.05 unless otherwise stated, and performed using MedCalc for Windows, version 19.0.7 (MedCalc Software).

3 | RESULTS

3.1 | Patient demographics

A total of 65 patients were screened for the study following a clinical diagnosis of HCC, of which 40 met eligibility criteria. Reasons for exclusion included a final pathological diagnosis other than HCC (n = 6), poor recovery post tumour resection (n = 9), unresectability (n = 3), withdrawal of consent (n = 2), diagnosis of a second cancer (n = 1) and development of metastatic disease (n = 4) (Figure S1). Table 1 summarizes the clinical characteristics of all patients who proceeded on to receive adjuvant gefitinib. The median age was 63 years (range, 44-80 years) with 20 patients (50%) ≥65 years old. There was a male predominance (90.0%). Thirty-two patients (80%) were positive for either hepatitis B (n = 27) or C (n = 2) or both (n = 3), and cirrhosis was present in half the patient cohort. The median Child-Pugh score was 6 (range, 5-8). None of the patients reported excessive alcohol intake. Vascular invasion was present in 17 patients (42.5%), while 19 patients (47.5%) had tumour sizes larger than 5 cm. Multifocal disease was present in seven patients (17.5%). All patients except two had R0 resection of the liver tumour as curative therapy, and all patients except three completed 6 months of adjuvant gefitinib.

3.2 | Survival analyses

At the time of analysis, 33 patients had developed recurrent disease, out of which 14 patients had died. With a median duration of follow-up of 4.5 years, the median RFS was 24 months. Median OS was not reached. An estimated 5-year OS of 59.4% was achieved while the 5-year RFS was 15.1% (Figure 1). Under univariate analysis (Table 2), Child-Pugh score and T-stage were statistically significant predictors for OS; vascular invasion and T-stage were significant predictors for RFS (Figure 2). Multivariate analysis (Table 3) demonstrated that high Child-Pugh score was a significant independent predictor for both OS (HR 2.87, 95% CI 1.36-5.60, P = .0050) and RFS (HR 1.74, 95% CI 1.01-3.00, P = .0471). Advanced T-stage (3-4) was also independently associated with worse OS (HR 4.97, 95% CI 1.57-15.75, P = .0064) and RFS (HR 2.82, 95% CI 1.28-6.25, P = .0105).

3.2.1 | Somatic mutational landscape in HCC

We performed whole exome sequencing of 33 HCCs with matched non-tumour liver tissues (Table S1) and identified a total of 7196 somatic mutations. A median of 105 silent and 115 non-silent mutations per tumour (range, 3-249) was observed. Somatic synonymous and non-synonymous variants of interest are represented in an oncoplot, including recurrent mutations in known HCC-associated genes such as TP53 and CTNNB1 (Figure 3). The estimated proportions of mutations in each of six possible base substitution classes, as well as the proportions of mutations contributed by each inferred mutational signature in individual HCC samples were examined. The signature for exposure to AA, as characterized by a majority of T:A > A:T mutations, was observed in 18 cases (signature 22, 54.5%; Figure 4; Figure S2). Tumour mutation burden (TMB) in AA...
signature-containing tumours (mean = 2.6 mutations/megabase) was higher than in non-AA tumours (mean = 1.9 mutations/megabase; \( P = .0483 \)). Other mutational patterns identified included signatures 1 (aging), 4 (tobacco smoking), 6 (defective DNA mismatch repair), 12, 16, 23 and 24 (aflatoxin). Analysis of somatic copy-number alterations identified six gained genomic regions (13q34, 19p13.11,
17q24.3, 2p25.1, 1q24.2 and 5p15.33). We further identified eight deleted regions (1p36.32, 4q21.22, 13q14.3, 11q24.1, 19p13.3, 6p21.31, 16p13.3 and 22q11.21; Figure S4). Gene-level copy-number analysis suggested \( \text{EGFR} \) amplification in 16 cases (Figure S5).

### 3.2.2 Correlation of molecular characteristics with survival outcomes

We examined if base substitution mutation classes correlated with survival outcomes. Across the entire subcohort with mutation data (Tables S1-S3), the values for \( \text{C} > \text{A} \) (median: 49, range: 3-108), \( \text{C} > \text{G} \) (median: 18, range: 0-45), \( \text{C} > \text{T} \) (median: 63, range: 5-133), \( \text{T} > \text{A} \) (median: 24, range: 1-124), \( \text{T} > \text{C} \) (median: 46, range: 3-96) and \( \text{T} > \text{G} \) (median: 15, range: 0-29) were dichotomized using optimized cut-offs to predict OS as derived from ROC curve analysis (Figure S3). High \( \text{T} > \text{A} \) mutation counts were associated with better OS (HR 0.29, 95% CI 0.09-0.90, \( P = .033 \)). Conversely, high \( \text{C} > \text{T} \) mutation counts were associated with worse OS (HR 4.55, 95% CI 1.20-17.31, \( P = .026 \)). Both variables retained independent significance in multivariate models incorporating Child-Pugh score and T-staging (Tables S4 and S5). Both high \( \text{T} > \text{A} \) and \( \text{C} > \text{T} \) mutation levels were correlated with TMB, though TMB by itself was not correlated with any survival outcomes (Figure 5; Figure S6). In terms of mutational signatures, AA-mutagenesis associated with improved RFS (HR 0.37, 95% CI 0.16-0.85, \( P = .019 \)) and we also observed a trend towards improved OS (HR 0.36, 95% CI 0.11-1.16, \( P = .088 \)) (Figure S6). In line with this, we noted that HCC without AA mutagenesis was associated with larger tumour size (\( P = .005 \)) and early recurrences or death within 2 years of surgery (\( P = .037 \); Table S3). AA mutagenesis was neither correlated with the presence of HBV or HCV infection, nor with patient factors such as age, sex or presence of cirrhosis. \( \text{EGFR} \) amplification did not affect survival outcomes significantly (Figure S6).

### 4 DISCUSSION

No proven adjuvant therapies for HCC following curative surgery currently exists. Contemporary guidelines do not recommend any adjuvant therapy, and patients either opt for close clinicoradiologic surveillance or clinical trials if available. Several attempts to address this unmet need have been unsuccessful, and most patients eventually develop recurrent or metastatic disease. Early trials evaluating the role of alpha-interferon, vitamin K2 and peretinoin have all failed to improve clinical outcomes.\(^{26-28}\) In the more recent phase 3 STORM trial evaluating the role of adjuvant sorafenib versus placebo for HCC after curative resection or local ablation, a median RFS of approximately 33 months was achieved in both groups, negating any significant treatment effect with sorafenib.\(^{3}\) In our pilot study on gefitinib as adjuvant therapy for HCC following successful curative surgery, the median RFS was 24 months. Although comparing favourably with earlier HCC trials, the benefit of adjuvant gefitinib is modest at best. The prognosis of this group of patients certainly remains dismal, and more efforts are required to overcome this problem. Current trials underway include the investigation of antiviral therapy with nucleotide analogues for HBV-related HCC, as well as several studies evaluating checkpoint immunotherapeutics.\(^{29,30}\)

Our present study examined the genomic landscape of 33 primary HCC samples obtained following liver resection from patients in Singapore and explored potential relationships with...
clinical outcomes. The mutational signature of exposure to AA was observed in over half the cases (55%), which is comparable with that observed in other Asian cohorts from Taiwan (78%) and China (47%).

In HCC with AA mutagenesis, the overwhelming majority of mutations are T:A > A:T transversions, with a significant strand bias as lesions occurring on transcribed strands may be identified and corrected by transcription coupled repair. The relative contribution of AA-related mutations to the entire tumour mutation load, however, is lower in our cohort as compared to Taiwan. The sale of products containing AA has been controlled by the Singapore Health Sciences Authority and AA has been regulated under the Poisons Act in Singapore since the early 2000s. However, raw herbs that may contain AA are not monitored by the Health Sciences Authority, and AA herbs and herbal products from overseas are easily purchased over the Internet. Nonetheless, with a significant number of people obtaining herbal products from

**FIGURE 4** Distinctive subsets of hepatocellular carcinoma (HCC) defined by mutational signatures. (A) The estimated proportions of mutations in each of six possible single base substitutions, as well as the (B) proportions of mutations contributed by each inferred mutational signature in individual HCC samples are as shown. AA exposure is represented by COSMIC signature 22.
unlicensed agencies, in particular online stores, AA-containing herbs and herbal products may still continue to pose a major health problem, and it will be prudent to increase public awareness of their potential harm. The clinical relevance of AA-related HCC is expected to persist in the near future as the prevalence of viral hepatitis continues to decline.\textsuperscript{32}

Interestingly, we observed that HCC with AA mutagenesis was smaller in tumour size and associated with a reduced incidence of early recurrences or death within 2 years of surgery. Correspondingly, there was a suggestion of improved survival outcomes for AA-mutagenized HCC. In addition, we found that high levels of T > A transversions were associated with better patient survival. Interestingly, AA-exposed upper tract urothelial carcinomas were also recently reported to have improved survival outcomes.\textsuperscript{34} A recent comprehensive report on the integrated proteogenomic characterization of primary resected HBV-related HCC (n = 159) described 35% of the cases harbouring AA signatures. Although the study did not show significant association with survival outcomes, AA signatures were correlated with favourable clinical features including the absence of tumour thrombus and earlier disease stages.\textsuperscript{34} In addition, our results and others have consistently shown that HCCs with AA signatures harbour greater tumour mutation burden.\textsuperscript{5,34} These tumours also demonstrate higher levels of predicted neoantigen load, significantly denser infiltrating CD8+ T cells, as well as higher expression of ICOS, OX40, PD-L1 and LAG3.\textsuperscript{34} Collectively, these findings across different HCC cohorts indicate that AA signatures may confer favourable prognostic characteristics, and HCC with AA mutagenesis may potentially respond to checkpoint immunotherapy.\textsuperscript{35}
In conclusion, this study revealed the potential clinical relevance of mutational signatures in resected HCC. We recognize the limitations of our pilot study precluding definitive evidence for the clinical utility of gefitinib in the adjuvant setting after curative resection of HCC and acknowledge that our findings remain to be confirmed in randomized controlled studies. Nonetheless, patient outcomes with gefitinib as adjuvant therapy after curative resection for HCC were modest, highlighting the need for further research.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
JYC analysed the data and drafted the manuscript; EL and JHH assisted with statistical analyses and data interpretation; CCN, JYL, VR, WL and SG processed tissue and performed sequencing experiments; AHL, AB and SGR performed the bioinformatic analyses; XX, LDB, CYC, AYFC, PCC, CKT, CKH, KHL, WWW, JLK, AC and GL obtained patient samples and data; BTT and AYC designed the study; JYC, SGR, BTT and AYC interpreted the results and revised the manuscript; and all authors read and approved the final version of the manuscript.

ETHICS APPROVAL AND PATIENT CONSENT
Written informed consent was obtained in accordance with the Declaration of Helsinki. The study is registered with ClinicalTrials.gov (NCT00282100) and approved by the IRBs of all participating hospitals.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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