Prognostic Biomarkers for Hepatocellular Carcinoma

Koh Rui Qi, Pek Mi Xue Michelle and Tan Min-Han

1 Background and Purpose of Research

Hepatocellular carcinoma (HCC) affects approximately half a million patients worldwide and is the most rapidly increasing cause of cancer death in the United States owing to the lack of effective treatment options for advanced disease [1]. Numerous lines of clinical and histopathologic evidence suggest that HCC is a heterogeneous disease, but a coherent molecular explanation for this heterogeneity has yet to be reported [2]. Due to the phenotypic and molecular diversity of HCC, it is a challenge to determine a patient's prognosis [3]. It would be ideal to increase monitoring of patients with poor prognosis. Thus the inability to accurately predict prognosis leads to excessive or insufficient time spent following patients, resulting in unnecessary anxiety and cost for patients, and inefficient allocation of resources for hospitals.

In clinical settings, prognostic assessment and decision of surgical treatment are based on one of the tumour staging systems (i.e. Barcelona Clinic Liver Cancer [BCLC], cancer of the liver Italian program, Japan Integrated Staging, and TNM) [4, 5] These different staging systems are based mainly on the tumor size, number of nodules, and severity of the liver disease [5]. Some authors have proposed to improve the staging system by introducing tumor biomarkers, such as the level of α-fetoprotein in serum and pathological features, like microvascular invasion and tumour differentiation [4, 6]. To refine prognosis scoring, the search of molecular biomarkers is an expanding field [7, 8]. More than 18 different molecular signatures have been published but few have been externally validated [7–11]. One of these validated molecular prognostic classifications was the G3 signature, which has been shown to be associated with tumour recurrence in both fresh-frozen and paraffin-fixed HCC [12, 13]. Interestingly, the G3 subgroup of HCC also showed the strongest association with tumor recurrence among 18 different molecular signatures [13].

K. R. Qi · P. M. X. Michelle · T. Min-Han (✉)
Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, the Nanos #07-01, Singapore 138669, Singapore
e-mail: mhtan@ibn.a-star.edu.sg

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Concerning the cancer field effect in cirrhosis, a 186-gene signature derived from non-tumour liver sample was also able to predict late recurrence and survival by capturing biological signals of aggressive phenotype from the underlying cirrhosis [7, 14].

A technical challenge facing the use of gene-expression profiling to predict the outcome of hepatocellular carcinoma has been the lack of suitable specimens from patients. Current methods of genome wide expression profiling require frozen tissue for analysis, whereas tissue banks with clinical outcome data generally have formalin-fixed, paraffin-embedded (FFPE) specimens. Even today, the vast majority of specimens are formalin-fixed; the collection of frozen tissues has yet to become routine clinical practice [7].

Therefore, a simple, easy to use test remains to be identified and endorsed in HCC clinical guidelines. We aimed to identify a molecular signature able to accurately predict prognosis of patients with HCC using FFPE samples, to enhance clinical decision making. Our study comprised of two parts: (1) identification of a 9 key gene markers in a training set of patients; (2) validation of our gene markers in an independent cohort.

2 Hypothesis

Potential HCC prognostic biomarkers can be validated through RNA extraction and cDNA conversion from FFPE samples to develop a multi-gene qPCR assay.

3 Materials and Methods

3.1 Study Population

The retrospective study was conducted with a cohort of 82 first-time HCC patients treated at Singapore General Hospital (SGH) between 2011 and 2012. All patients had histologically confirmed HCCs for which FFPE primary tumor blocks were available. In the initial stage of study, prognostic genes were identified by my mentor based on prior microarray studies on frozen tissue samples of 23 patients (Fig. 1). The coefficients of variance (standard deviation/mean) of gene expression were calculated from microarray data. Three genes (PSMB2, RPS18, MRPL30) whose expression was the least variable were identified to serve as normalization genes for qPCR. Primers for the 9 potential prognostic transcripts selected for assay development and the 3 normalization genes were then provided by my mentor and used as received. In the second stage of study, an independent set of 82 FFPE samples were used for

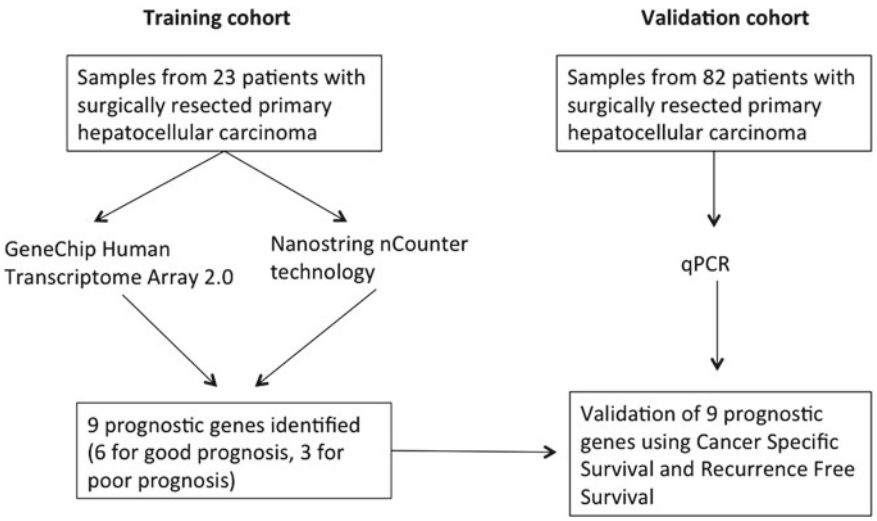


Fig. 1 Study design. In the training cohort, 2 microarray studies were used to select 9 potential prognostic genes. Filtering was done to keep the exons that displayed similar correlation to survival in the 2 assays. These 9 genes were then validated for their accuracy in predicting survival

Fig. 2 Study design for validation cohort

validating the performance of these prognostic genes and developing a multi-gene qPCR assay (Fig. 2). Clinical and pathological data were obtained from ongoing chart review of medical records and electronic databases.

3.2 RNA Extraction from FFPE Tissue

FFPE blocks were sectioned in 5-μm sections and stained with hematoxylin-eosin for confirmation of histological diagnosis and tumour tissue content. For each sample, 1–3 FFPE sections were deparaffinated and microdissected with a sterile single-use scalpel to obtain tumour-specific parts. RNA was then extracted using RNeasy FFPE Kit (Qiagen, Hilden, Germany).

3.3 Design of qPCR Assay for FFPE Tissue RNA

1 μg of extracted RNA was reverse transcribed with random hexamer primers using High Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative expression of each target gene was measured by real-time qPCR with Power SYBR Green Master Mix (Life Technologies) on a CFX96 machine (Bio-Rad Laboratories, Hercules, CA, USA). 12.5 ng of the four-fold diluted cDNA was used as template in a 10 μl reaction with primers at a final concentration of 200 nM. PCR amplicons were checked for specificity of amplification with melt curve. Negative controls were run for each plate.

3.4 Processing of qPCR Expression Data

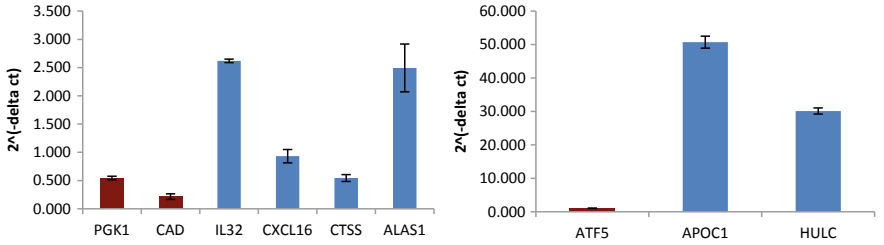
We designed qPCR assays for a set of 12 genes (3 reference, 9 prognostic genes) identified from prior microarray studies. qPCR expression data collected as cycle threshold (Ct) expression was normalized by subtracting Ct values from the geometric average of Ct values for three normalization genes. The delta Ct value was then converted to a linear scale by the function $2^{\wedge -\text{delta Ct value}}$ to obtain the gene profile of the 9 genes: PGK1, CAD, ATF5, APOC1, IL32, HULC, CXCL16, CTSS and ALAS1.

3.5 Statistical Analysis

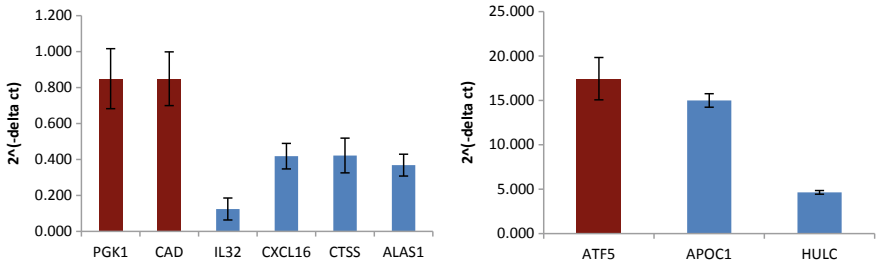
Using-delta Ct value for the 9 genes as input, K means analysis was performed using "fpc" package in R. Patients were clustered into 2 groups based on their levels of gene expression. Survival analysis was performed using "survival" package in R. The package was then used to evaluate the association of 9-gene prognostic signature with cancer-specific survival and relapse-free survival, and significance was determined by the log-rank test.

4 Results and Discussion

The 6 protective genes identified from the prior microarray studies were APOC1, IL32, HULC, CXCL16, CTSS and ALAS (blue). The 3 adverse genes identified were PGK1, CAD and ATF5 (red). Graphs 1 and 2 show samples with gene expression indicating good and poor prognosis respectively. A patient with good prognosis shows a relatively higher level of expression of protective genes compared to adverse genes



Graph 1 Example of patient with good prognosis



Graph 2 Example of patient with poor prognosis

(Graph 1). Conversely, a patient with poor prognosis shows a relatively higher level of expression of adverse genes compared to protective genes (Graph 2).

In our statistical analysis, the 82 patients were grouped into two clusters according to their levels of gene expression. We found our 9-gene assay to be significantly correlated with relapse-free survival (RFS) ($p = 0.0493$) (Fig. 3a), which includes patients who died of disease and those with disease recurrence. Patients were clus-

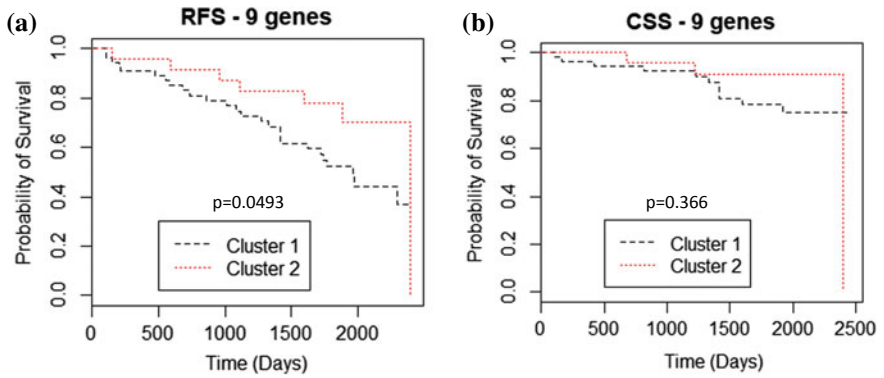


Fig. 3 Survival curves for relapse-free survival (**a**) and cancer specific survival (**b**) according to the level of expression of the 9 prognostic genes among the 82 patients

tered into 2 groups based on their distinct gene expression profiles. Clustering was found to be associated with RFS. However, when we tested the 9-gene assay for cancer specific survival (CSS), which includes only patients who died of disease, there was no statistical significance ($p = 0.366$) (Fig. 3b). The possible reason for the difference observed between CSS and RSS is the high probability of survival when patients with recurrence are identified and treated early. This further justifies the close monitoring of patients with poor prognosis. Our results support the validity of our assay in predicting the probability of relapse-free survival in HCC patients.

4.1 Clinical Application

We describe here a practical 9-gene assay capable of predicting the prognosis of HCC patients. The samples obtained from surgeries were FFPE tissue. Accordingly, our assay has been developed using RNA extracted from FFPE materials from surgeries and thus is expected to perform on such material in the clinical setting. The utility of the assay in abundantly available, routinely collected FFPE material greatly broadens the scope for rapid validation. Our assay can also be expanded to work on FFPE samples obtained from pre-operative core biopsies.

4.2 Cellular Functions of Prognostic Genes

The 9 genes in the prognostic assay—PGK1, CAD, ATF5, APOC1, IL32, HULC, CXCL16, CTSS and ALAS1—represent genes for angiogenesis, cell proliferation, transcription regulation, monocyte differentiation in the liver, chemokine signaling, MHC class II presentation and heme biosynthesis. The unbiased selection method in the 2 essays likely accounts for the wide variety of cellular functions encompassing in the prognostic gene set.

4.3 Limitations

The limitations of this study are its retrospective design, incomplete follow-up information of patients and the relatively limited number of subjects with poor prognosis for the validation cohort. Many patients with disease relapse survived for relatively longer than expected. This may have resulted in some difficulty in clustering data according to survival, resulting in the lack of statistical significance in the CSS value. External validation in prospective trials will be crucial to determine clinical value. Prognostic signatures ideally should be considered alongside optimal clinical predictors of outcome, such as the Barcelona Clinic Liver Cancer (BCLC), cancer of the

liver Italian program, Japan Integrated Staging, and TNM. Future systematic studies will be important to address this.

Furthermore, the difference in level of protective genes and adverse gene expression in HCC is less distinct compared to that in other types of cancers. As it is more challenging to distinguish high gene expression from low gene expression, careful optimization and external validation in a larger cohort will be needed to further ensure the reliability of this assay. Additionally, predicting the survival for HCC patients is especially difficult because the liver plays crucial roles including detoxification, regulation of glycogen storage, plasma protein synthesis and hormone production, and is thus vital for the function of the human body. Many patients with HCC also have underlying fatal conditions such as cirrhosis and hepatitis B or C infection. In our study, it was difficult to ascertain whether the cause of death of certain patients was HCC or an underlying liver dysfunction. In a clinical setting, it may also prove challenging to predict survival of patients due to the plethora of other liver complications they may have in addition to HCC.

5 Conclusion and Recommendation for Future Work

In the future, the usefulness of our molecular 9-gene assay could be tested in clinical decision guidance. First, the 9-gene assay could be used to stratify the effectiveness of adjuvant therapy for various patients. This allows for more targeted and customized treatment that reduces cost and increases efficacy. Furthermore, the 9-gene assay could also modify transplantation indication, for example, by extending the Milan criteria to good molecular prognosis tumours even if it is >5 cm, whereas bad-prognostic molecular tumours within the Milan criteria could be excluded from liver transplantation or subjected to a more aggressive neoadjuvant strategy [15]. Despite the limited treatment options after liver resection in routine clinical practice, our 9-gene assay could also be tested to stratify the risk of relapse and death after liver resection in adjuvant randomized trial [16–18].

In conclusion we have designed a practical FFPE gene expression assay to predict the prognosis of HCC patients, with potential implications for therapeutic response. Incorporating the results from our assay allows an additional tool that can be integrated into the decision-making process, enhancing precision especially when it affirms the pathological assessment on core biopsy. We envision the use of this test to identify the patients with poorest prognosis in HCC to target intensive clinical follow-up and for predicting outcome and response to treatment.

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