

Original research

Unannotated small RNA clusters associated with circulating extracellular vesicles detect early stage liver cancer

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ABSTRACT

Objective Surveillance tools for early cancer detection are suboptimal, including hepatocellular carcinoma (HCC), and biomarkers are urgently needed. Extracellular vesicles (EVs) have gained increasing scientific interest due to their involvement in tumour initiation and metastasis; however, most extracellular RNA (exRNA) blood-based biomarker studies are limited to annotated genomic regions.

Design EVs were isolated with differential ultracentrifugation and integrated nanoscale deterministic lateral displacement arrays (nanoDLD) and quality assessed by electron microscopy, immunoblotting, nanoparticle tracking and deconvolution analysis. Genome-wide sequencing of the largely unexplored small exRNA landscape, including unannotated transcripts, identified and reproducibly quantified small RNA clusters (smRCs). Their key genomic features were delineated across biospecimens and EV isolation techniques in prostate cancer and HCC. Three independent exRNA cancer datasets with a total of 479 samples from 375 patients, including longitudinal samples, were used for this study.

Results ExRNA smRCs were dominated by uncharacterised, unannotated small RNA with a consensus sequence of 20 nt. An unannotated 3-smRC signature was significantly overexpressed in plasma exRNA of patients with HCC ($p<0.01$, $n=157$). An independent validation in a phase 2 biomarker case-control study revealed 86% sensitivity and 91% specificity for the detection of early HCC from controls at risk ($n=209$) (area under the receiver operating curve (AUC): 0.87). The 3-smRC signature was independent of alpha-fetoprotein ($p<0.0001$) and a composite model yielded an increased AUC of 0.93.

Conclusion These findings directly lead to the prospect of a minimally invasive, blood-only, operator-independent clinical tool for HCC surveillance, thus highlighting the potential of unannotated smRCs for biomarker research in cancer.

Significance of this study

What is already known on this subject?

► Current surveillance tools for the detection of early stage HCC are suboptimal. EV-based early detection biomarkers have historically been plagued by poor reproducibility and are biased towards studying well-characterised micro RNA (miRNA) across multiple indications.

What are the new findings?

► Using a novel approach to reproducibly quantify and characterise the largely unexplored landscape of unannotated small RNA expression signatures from payloads of circulating EVs, we identified unannotated biomarkers capable of detecting early stage HCC with high accuracy.

How might it impact on clinical practice in the foreseeable future?

► These findings directly lead to the prospect of a minimally invasive, blood-only, operator-independent hepatocellular carcinoma surveillance biomarker.

INTRODUCTION

Extracellular vesicles (EVs), including microvesicles and exosomes, are nanoparticles whose nucleic acid payload is capable of priming receptor cells to modify key cellular functions.^{1,2} EVs are heterogeneous, both in terms of biogenesis and content.³ While larger EVs such as apoptotic bodies mostly contain fragmented DNA, smaller EVs such as exosomes are enriched in non-coding, regulatory small RNAs.^{2,4} In cancer, EVs are increasingly recognised as key players in tumour initiation and metastasis, mainly through micro RNA (miRNA) trafficking, prompting their evaluation as early detection and treatment response biomarkers.⁵ Importantly, most



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studies characterising extracellular RNA (exRNA) and studying EV-related biomarkers apply conventional, reference-based, RNA sequencing approaches, and are thus limited to known annotated genomic regions (eg, miRNA, small nucleolar RNA (snoRNA), long non-coding RNA (lncRNA), etc). However, small RNAs arise from thousands of endogenous genes and are part of the genomic ‘dark matter’ of highly abundant yet largely uncharacterised non-coding RNA, with emerging roles in regulating gene expression via post-transcriptional and translational mechanisms. In fact, relatively little attention has been paid to characterising the general expression landscape of circulating EV small RNA and their precursors in this context regardless of biotype, especially for those expressed from unannotated genomic regions.

With a 5-year survival of 18%, liver cancer is the second-most lethal malignancy after pancreatic cancer. Projections estimate more than 1 million deaths due to this cancer in 2030 worldwide.⁷ Survival in patients enrolled in early detection programmes of hepatocellular carcinoma (HCC), the most common form of primary liver cancer, doubles that of those not enrolled in surveillance.⁸ However, implementation of surveillance among patients at high risk of HCC in the USA is very low (20%)⁹ and the performance of recommended surveillance tools (ie, ultrasound and serum alpha-fetoprotein (AFP))^{10 11} is suboptimal, with low sensitivity (63%) and moderate specificity (83%) for early stage HCC missing close to 40% of tumours.¹² Improvement in this area is urgently needed by developing better read-outs of oncogenesis and facilitating implementation of surveillance through minimally invasive, operator-independent tools.

Our aim was to characterise the small exRNA expression landscape associated with circulating EVs, to identify novel small RNA biomarker candidates, and to test their clinical utility for the detection of early stage HCC among high-risk patients. Strongly departing from previous exRNA characterisation studies, which are restricted to quantifying expression of known (ie, annotated) transcripts,^{13 14} we adopt a different approach by de novo assembly and characterisation of the small RNA expression landscape of exRNA, specifically including unannotated genomic regions across an HCC plasma EV dataset and a prostate cancer dataset with tumour and adjacent tissue, urine and blood specimens. The latter dataset was used to define discrete loci called small RNA clusters (smRCs), delineate their key genomic properties and quantify their reproducibility across biofluid and isolation techniques. In the second part of our study, two independent HCC cohorts were used to identify and validate potential candidates to discriminate early stage HCC and controls at high risk in the setting of a phase 2 biomarker case-control study. In summary, by using whole RNA sequencing of EV-associated exRNA, we describe novel clinically relevant smRCs in circulating exRNA as an unrecognised source for biomarker discovery. In our study, a 3-smRC signature was able to discriminate between patients with early stage HCC and patients at high risk for HCC with better performance compared with a meta-analysis on the current standard of surveillance by ultrasound±AFI.¹²

MATERIALS AND METHODS

Patient enrolment and study cohorts

This study uses three independent cancer exRNA datasets (figure 1B).

1. A prostate cancer cohort, which we termed the ‘smRC characterisation’ cohort (n=9 patients, total of 41 samples): this

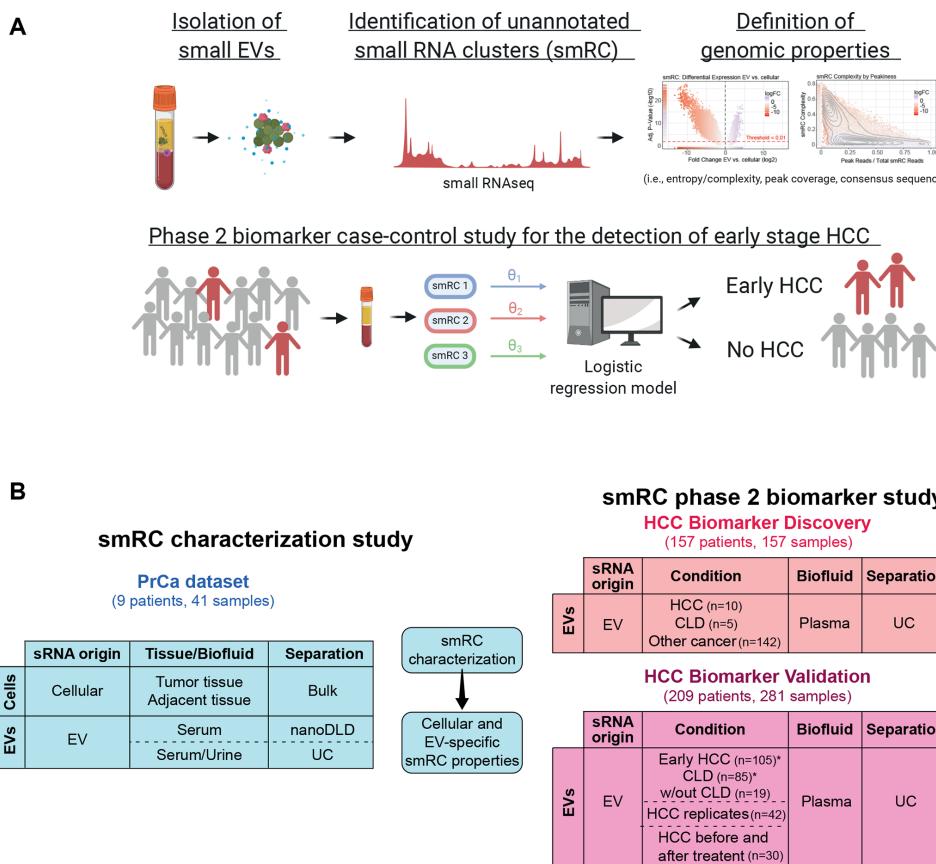
cohort served to define and study the properties of smRCs in exRNA. For this dataset, de-identified data and biospecimens from human subjects consented under ongoing institutional review board (IRB) approved protocols at the Icahn School of Medicine at Mount Sinai (GCO# 14–0318, 15–1135 and 10–1180) were collected from patients with prostate cancer (PrCa) undergoing prostatectomy. Specifically, biospecimens included prostate cancer and adjacent prostate non-tumorous tissue from biopsy or prostatectomy, urine and serum, where applicable. Each of these protocols involves the prospective collection of clinical data (eg, demographics, baseline characteristics, treatments and outcomes).

2. A HCC ‘biomarker discovery’ cohort (n=157 patients, total of 157 samples): to identify differentially expressed smRCs between patients with HCC and controls with chronic liver disease (CLD), and patients with non-HCC malignancies to test the HCC specificity of our biomarkers.
3. An independent HCC ‘biomarker validation’ cohort (n=209 patients, total of 281 samples, including 42 patients with replicates to assess assay reproducibility and 30 patients with samples before and after HCC treatment): to confirm their clinical utility in a phase 2 biomarker case-control study for detection of early stage HCC.

Samples for the HCC ‘biomarker discovery’ and ‘biomarker validation’ cohorts were collected from consented patients enrolled in an IRB-approved protocol to derive new HCC biomarkers from blood (HS-15-00540) or provided by the Tisch Cancer Institute Biorepository (HSM#10-00135) at the Icahn School of Medicine at Mount Sinai. Thus, HCC cases and controls were collected from the same clinical setting for the biomarker discovery and validation cohorts. Importantly, for the HCC biomarker discovery cohort, cases and controls were matched for age, gender, presence of cirrhosis and aetiology (online supplemental table S1). Small RNA sequencing data from patients with other (non-HCC) malignancies were downloaded from exRNA atlas (<https://exrna-atlas.org/>, RRID:SCR_017221, including n=100 colon cancer, n=6 pancreatic adenocarcinoma and n=36 patients with prostate cancer, respectively).

For the phase 2 biomarker case-control validation study, we included three patient populations: (1) HCC cases limited to very early or early stage patients according to the Barcelona Clinic Liver Cancer (BCLC) classification⁷ (ie, stages 0 or A). All patients with HCC were treatment-naïve at the time of blood sampling, (2) patients with liver cirrhosis or different forms of CLD at high risk for HCC as per clinical practice guidelines,^{10 11} but without radiological evidence of HCC at the time of blood collection and (3) patients with benign liver nodules (eg, haemangioma) without CLD. However, the latter were not considered in the logistic regression model. HCC diagnosis was made according to the criteria of the European Association for the Study of the Liver.¹¹ In a subset of patients (n=30), sequential blood samples were available before and after these patients had received HCC treatment. Response was assessed according to modified Response Evaluation Criteria In Solid Tumors (mRECIST).¹⁵ Liver cirrhosis was diagnosed based on histology, or non-invasively through combined transient elastography, imaging or laboratory evidence of liver dysfunction and portal hypertension. Patients with concurrent malignancies were excluded.

Methods and supplementary data on sample collection and enrichment of EVs from human plasma, serum and urine, characterisation of EV-enriched isolates, RNA extraction, small library preparation and next-generation sequencing, trimming, alignment, deconvolution analysis, smRC definition and properties,



*These samples were included in the phase 2 biomarker case-control study to validate the 3-smRC early HCC detection signature.

Figure 1 Study summary and flow chart for sample distribution. (A) SmRCs from unannotated genomic regions were identified by unsupervised small RNA sequencing from circulating EVs and characterised. Their clinical utility was confirmed in a phase 2 biomarker case–control study for the detection of early stage HCC (created with BioRender.com). (B) Schematic view of study flow diagram with different cohorts, and available specimen and separation method for each cohort. Three independent datasets with a total of 479 samples from 375 patients were included. CLD, chronic liver disease; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; nanoDLD, integrated nanoscale deterministic lateral displacement arrays; PrCa, prostate cancer; smRCs, small RNA clusters; UC, differential ultracentrifugation.

HCC smRC biomarker selection, reverse transcription quantitative PCR (RT-qPCR), smRC overlap with known biotypes and prostate cancer motif sequences can be found in the online supplemental material.

Data analysis

Following the guidelines of the Early Detection Research Network (EDRN) by the National Cancer Institute and the white paper on biomarker development in cancer,¹⁶ we conducted a phase 2 biomarker case–control study for early detection of HCC. This set of guidelines is generally used by academic and industry researchers who conduct biomarker studies and has been adopted by the US Food and Drug Administration as the benchmark for approval of new diagnostic devices. According to this paper, a phase 2 biomarker study is a retrospective case–control study to estimate the true positive rates and false positive rates or area under the receiver operating curve (AUC) for the clinical biomarker assay and to assess its ability to distinguish subjects with cancer from subjects without cancer. Although the primary aim of this study was to assess the performance of our novel early HCC detection biomarker test, we wanted to put our results into clinical context by comparing them to the current gold standard for early HCC surveillance (ie, abdominal ultrasound and AFP).^{10 11} Based on the largest meta-analysis on surveillance for HCC, sensitivity of ultrasound and AFP is 63%

for early stage tumours with a specificity of 83%.¹² We powered this study to detect an increase in sensitivity from 63% to 80% and specificity from 83% to 95% when comparing ultrasound and AFP to our new test. Given an α of 0.05 and a power ($1-\beta$) of 90%, the number of samples needed to detect this difference based on asymptotic normal distribution theory¹⁷ was 89 cases (early HCC) and 83 controls (patients at high risk of HCC and CLD) (simulations using different exact or approximate CIs for the difference of binomial proportions¹⁸ resulted in 93 cases and 82 controls; online application: https://mwsill.shinyapps.io/sample_size_diagnostic_test/).

The analysis of the phase 2 biomarker case–control study to test the performance of the 3-smRC signature for early detection of HCC was limited to early stage HCC (n=105) and controls at risk for HCC (CLD, n=85) to represent the optimal population of interest.^{11 16} We used penalised maximum likelihood techniques, bootstrap and cross-validation to estimate and control for model optimism, RT-qPCR batch plate effects and overfitting, and also rigorously computed the positive and negative predictive power estimates of our 3-smRC early detection signature. We computed a number of indices of model performance, discrimination measures and calibration measures under bootstrap resampling (n=1000), extended in online supplemental table S2, in order to demonstrate model performance and estimate generalisation error by averaging performance

across bootstrap resampling. The key measure of discrimination, Somers' D_{xy} , is the rank correlation between the observed and predicted response values, which in the case of logistic regression for a binary response reduces to simply $D_{xy} = 2(c-1/2)$, where c is Harrell's c-statistic and equal to the AUC of the receiver operating curve (ROC) for the early HCC vs CLD prediction. In the case of the smRC model, we immediately deduce that the bootstrap adjusted AUC is $1/2 + 3/8 = 7/8 = 0.875$. Modest adjusted modified $R^2 \sim 0.52$ is observed, combined with bootstrap-adjusted slope and intercept indicating modest and acceptably low overfitting. Relatively bootstrap-adjusted low E_{max} (the maximum error in predicted probabilities), modest Brier score (B), very low unreliability index (U), high discrimination (D), high quality ($Q=D-U$) also indicate a reasonably robust model. Also, the bootstrap adjusted total Gini's mean difference based on the smRC model is a healthy 2.44, which robustly represents typical log-odds differences between patients with early HCC and patients with CLD predicted by the model. Converting this early HCC log-odds estimate to an early HCC probability prediction, we see that the typical predicted probability gap between patients with early HCC and patients with CLD is 38%. Finally, we compute the partial mean Gini's scores of the smRC model predictors and find that the smRCs themselves have by far the largest termwise log-odds compared with any technical variance covariates (eg, batch). We note in passing that repeated cross-validation gave similar results for D_{xy} and adjusted slope (extended in online supplemental table S2).

We next repeated the penalised maximum likelihood estimation procedure using a model with both smRCs and AFP readings included, given that a log likelihood ratio test for an AFP term was highly significant ($p < 1e-8$). Computing the same indices of model performance across bootstrap resampling ($n=1000$), we found dramatically better performance for a combined model, including our 3-smRC signature and AFP compared with the 3-smRC signature alone, with bootstrap adjusted AUC ~ 0.93 , lower overall error and evidence for overfitting, a much smaller B 0.11, and a dramatic increase in the Gini's indices such that a typical early HCC–CLD predicted probability difference was 43%. Finally, even though balanced accuracy is not a proper scoring rule, we estimated the maximised balanced accuracy landscape by subjecting the smRC logistic regression model for HCC risk to a cross-validation repeated 1000 times (ie, a random 85% training and 15% testing split repeated 1000 times) and computing maximising sensitivity and specificity on the test ROCs.

For descriptive statistics, continuous variables are reported as median and categorical variables as counts and percentages. We used the Fisher's exact test and the Student's t-test to compare differences between categorical and continuous variables, respectively. Pearson's or Spearman's correlation coefficients were computed for correlation of continuous variables as indicated. Boxplot centre line shows median, box limits show upper and lower quartiles, whiskers show $1.5 \times$ IQR and points represent outliers. Error bars represent the 95% CIs. All statistical analyses were conducted on R studio (R V.3.5.0, RRID:SCR_000432).

RESULTS

Our study is summarised in figure 1A. It uses three independent cancer exRNA datasets (figure 1B): (1) a prostate cancer cohort, which we termed the 'smRC characterisation' cohort, to define and study the properties of smRCs in exRNA ($n=9$ patients, total of 41 samples). (2) A HCC 'biomarker discovery' cohort ($n=157$ patients) to identify differentially expressed

smRCs between patients with HCC and controls with CLD, and patients with non-HCC malignancies to test the HCC specificity of our biomarkers. (3) An independent HCC 'biomarker validation' cohort ($n=209$ patients, total of 281 samples, including 42 patients with replicates and 30 patients with longitudinal samples before and after HCC treatment) to confirm their clinical utility in a phase 2 biomarker case-control study for detection of early stage HCC. In total, our study included 479 samples from 375 patients.

Characterisation studies confirm enrichment of small EV from blood and urine

In all cohorts, differential ultracentrifugation (UC) was employed to enrich for EV isolates, and quality assessment of EV isolates was guided by recommendations of the International Society of Extracellular Vesicles.¹⁹ Specifically, we used transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), immunolabeling with western blotting for intracellular (ie, tumour susceptibility gene 101 protein, TSG101) and Exoview for transmembrane (ie, tetraspanins CD9, CD63 and CD81) vesicle proteins in a subset of samples (figure 2). This suggested an enrichment for small EVs (median size of 120 nm on NTA) with compatible morphology on NTA and TEM (figure 2A–C), and expression of typical markers for small EV populations with a dominance of CD9/CD81 and CD9/CD63 coexpression, and a paucity of CD63/CD81 coexpression (figure 2D–F). Additionally, for the 'smRC characterisation' cohort in prostate cancer, we isolated EVs from a subset of patients ($n=5$) using the integrated nanoscale deterministic lateral displacement 'lab-on-chip' technology (nanoDLD)²⁰ for serum samples. We also isolated purely cellular small RNA (<300 nt) from prostate cancer and adjacent non-cancerous tissue of the same patients to quantify exRNA isolation technology, biofluid and exRNA-specific variance in small RNA profiles, respectively. Part of our prostate cancer dataset has been included in an exRNA-atlas-based deconvolution analysis published earlier.⁴ In that study, an independent analysis found that our UC and nanoDLD isolation methods specifically isolate low-density (cargo type 1) and variable-density (cargo type 4) vesicles with minimum contamination from lipoproteins (cargo type 2) and argonaute proteins (cargo type 3B).⁴ For this study, we have now performed the same computational deconvolution analysis for our HCC 'biomarker discovery' dataset to determine carrier types and found that cargo type 4 was preferentially enriched (online supplemental figure S1A). In fact, cargo type 4 is associated with vesicles in the 60–150 nm size range, which were purified consistently with nanoDLD, and also the lowest-density OptiPrep fractions 1–3 from serum and plasma.⁴ Cargo type enrichments associated with low-density vesicles, lipoproteins, argonaute-2 (AGO2)-positive ribonucleoproteins (RNPs) and AGO2-negative RNPs were significantly depleted (online supplemental figure S1). Together, these results confirm a successful enrichment of small EVs from a variety of biospecimens of prostate cancer and patients with HCC with our methods.

Identification and characterization of smRCs from unannotated exRNA

In the HCC 'biomarker discovery' cohort, we detected recurrent small clusters of contiguous genomic regions with sufficient alignment coverage in unannotated regions. Normally, we would disregard them as part of our standard RNA sequencing analytical pipeline. However, these small RNA clusters (termed 'smRCs') presented with a dominant peak sequence on many

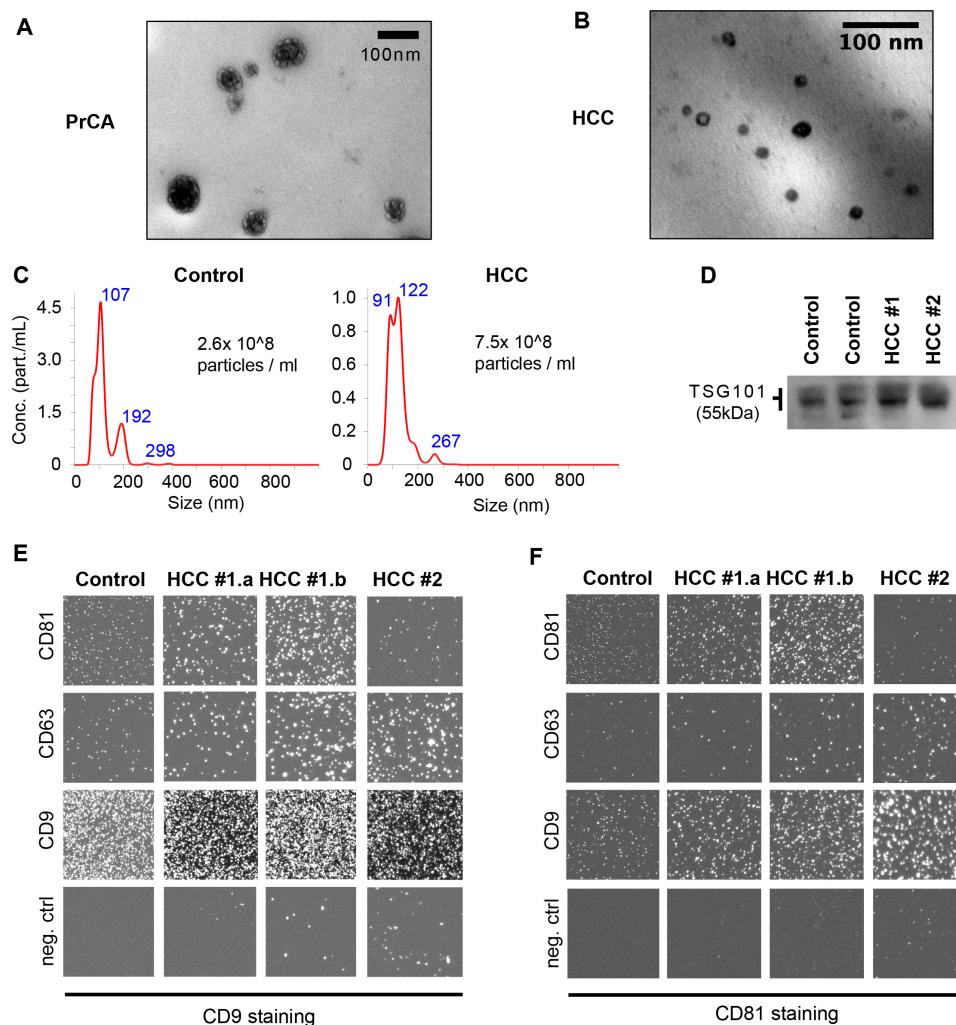


Figure 2 Quality assessment of EV enrichment process for exRNA extractions from human blood samples. (A and B) TEM image of prostate cancer serum isolate (A) and HCC plasma isolate (B). (C) Nanoparticle tracking analysis (Nanosight) results in the plasma isolate of a control (left) and patient with HCC (right) with corresponding size distribution and estimated particle concentration. (D) Western blotting image of protein lysate from isolate against TSG101 (~55 kDa) in two controls (left) and two patients with HCC (right). (E and F) Immunolabeling of the isolate with Exoview. Isolates were captured by indicated antibodies (CD81, CD63, CD9 and control IgG) on a chip and stained with CD9 (E) or CD81 (F) antibodies to visualise different EV subpopulations in one control and three HCC samples (#1.a and #1.b represent technical replicates from the same patient). EV, extracellular vesicle; exRNA, extracellular RNA; HCC, hepatocellular carcinoma; PrCa, prostate cancer; TEM, transmission electron microscopy; TSG101, tumour susceptibility gene 101 protein.

occasions, suggesting a non-random pattern, which prompted us to further investigate their genomic properties and potential role as cancer biomarkers. First, we captured the known heterogeneous genome-wide expression of clusters of small RNA precursors,²¹ each of which can give rise to multiple functional small RNA products, by defining clusters of small RNA reads (ie, smRCs, figure 3A). Adjacent smRCs are merged if they overlap within a minimal padding threshold (75 bp), and we define the key properties of smRCs: (1) entropy (ie, read tiling efficiency or complexity), (b) peak coverage and (c) consensus sequence of each smRC (see below). The set of all smRCs, computed once for all samples, is essentially the paired set of all accumulation loci of small RNA expression and their peak-coverage consensus sequences, and constitutes a smoothed, de novo assembled small RNA expression landscape with a standard count matrix.

To determine whether smRCs were specific to blood or present in other compartments and tissue types, we expanded our analysis and delineated key genomic properties of smRCs in our ‘smRC characterisation’ prostate cancer dataset, where

we had access to different biological sample types (blood, urine, tumorous and non-tumorous adjacent tissue) and different EV isolation methods (UC and nanoDLD^{22 23}). In order to profile the maximal coverage and overall distribution of expression within smRCs associated with exRNA, we defined two quantities: first, a ‘peak’ coverage, which is simply the ratio of reads in the smRC peak to total smRC coverage, and second, a tiling complexity measure, which is the ratio of unique read nucleotide sequences to total smRC coverage. Since almost all small RNAs arise from post-transcriptional processing of larger RNA precursors, the quantification of alignment patterns is largely an empirical task for which measures of maxima (peak coverage) and heterogeneity (tiling complexity) become crucial tools to classify these patterns. SmRCs with low tiling complexity are those with a non-uniform tiling of transcripts and a relative dominance of equal reads forming a peak. Here, the term peak-coverage consensus sequence is referring to the sequence of the dominant transcript (left panel of figure 3A). In contrast, smRCs with high tiling complexity are clusters of transcripts with a uniform tiling

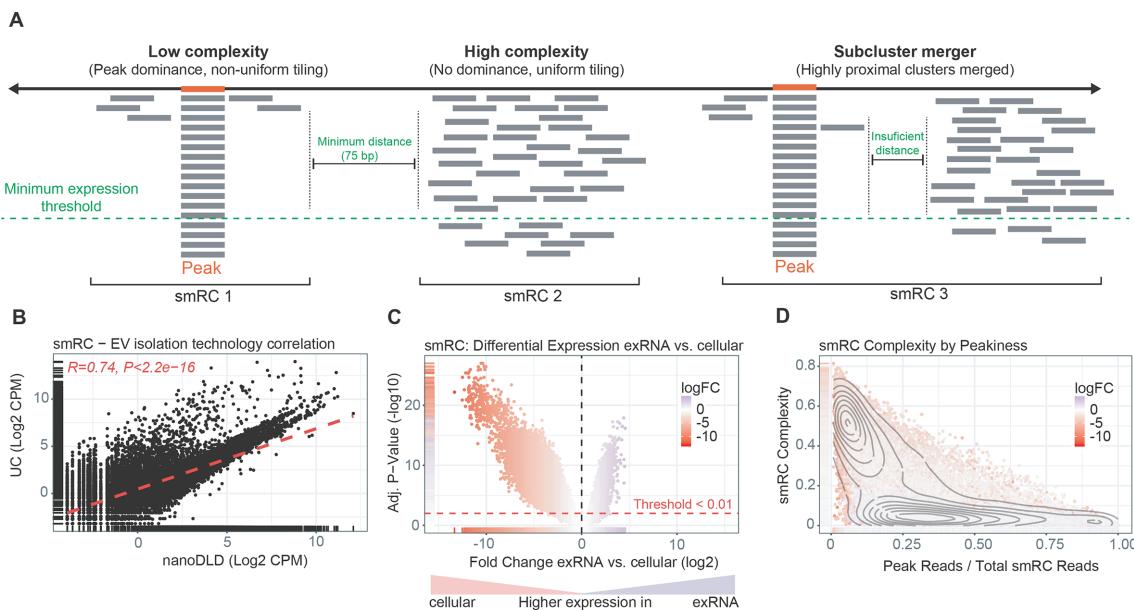


Figure 3 Key properties of smRCs. (A) Minimum coverage and subread length minimal spacing define smRCs. Read tiling complexity captures heterogeneity of smRC read distribution. (B) Correlation of smRC expression across different EV enrichment methods (ie, UC and nanoDLD). (C) Volcano plot for differential expression between smRC of cellular vs exRNA origin. (D) SmRC complexity as a function of peak coverage coloured by differential smRC expression between cellular and exRNA origin. SmRCs enriched in exRNAs (higher logFC, coloured towards purple) present with low complexity and higher peak coverage, whereas cellular smRCs (red) are more frequently of high complexity and lower peak coverage. EV, extracellular vesicle; exRNA, extracellular RNA; smRCs, small RNA clusters; UC, ultracentrifugation.

of reads and few peaks, which can result in a fairly long genomic region covered by this cluster (middle panel of figure 3A). The mean length of the consensus peak sequence was 20 nt (ranging from 15 nt to 100 nt in length). In contrast, the mean genomic length of smRCs was 674 nt (online supplemental figure S2A). Technical reproducibility of smRC quantification included comparing two different EV enrichment methods in serum (UC and nanoDLD), and different biofluid compartments (urine and serum) of the same patients. We found a high correlation between enrichment methods (Spearman's $r \sim 0.74$, $p < 2.2e-16$, figure 3B) with over 80% of smRCs detected by both methods above the 20th percentile of expression (online supplemental figure S3A). We found a modest correlation between different biofluid compartments (ie, urine and serum) using UC (Spearman's $r \sim 0.45$, $p < 1e-16$, see online supplemental figure S3B,C for self-reproducibility).

Taken together, we robustly identified smRCs, including in unannotated regions, across biospecimens and enrichment methods by an unsupervised data-driven view of the entire small RNA landscape.

ExRNA smRCs are enriched for non-coding transcripts from unannotated regions

Well-expressed smRCs possessed a heteroscedastic count variance profile, which facilitated usual differential expression analysis via linear modelling (online supplemental figure S2B). The total number and magnitude of overexpressed smRCs in cells were significantly higher than in exRNA (figure 3C). However, we observed a significant difference in the complexity of smRCs found in exRNA compared with cells, and found that the major contributor of smRC variable expression was RNA origin (with low complexity typical in exRNA vs high complexity typical of cellular smRC origin, online supplemental figure S2C, figure 3D). Indeed, the bimodal pattern reveals a clear separation between cellular smRCs, which overwhelmingly have relatively high

tiling complexity, and exRNA smRCs that have much stronger evidence for high relative peak coverages and low complexity. The mean size of the peak within smRCs was slightly higher than the minimal trimmed read length, and was significantly different between exRNA derived and cell derived (16.5 nt vs 22.6 nt, $p < 1e-16$). ExRNA-associated smRCs preferentially overlap unannotated small RNA species compared with cellular smRCs (online supplemental figure S4 and table S3). Finally, we orthogonally validated the expression of three unannotated smRCs from the prostate cancer dataset by correlating RNA sequencing data with RT-qPCR (online supplemental figure S3D and table S4).

These data demonstrate that EV-associated smRCs predominantly present with a small number of highly covered peaks (ie, low complexity and high peak coverage) compared with cellular-derived smRCs. They preferentially capture non-coding small RNA compared with protein-coding RNA but are also significantly enriched in unannotated genomic regions.

Identification of an HCC-specific 3-smRC signature in plasma exRNA

Given their high biological and technological independent reproducibility, tractable statistical properties and unique ability to discriminate concentrations of exRNA-specific small RNA, we computed the smRC profile of our 'HCC biomarker discovery' cohort of 15 patients, including 10 patients with HCC and 5 controls at risk for HCC matched for age, sex and aetiology of the underlying liver disease (online supplemental table S1). We found that exRNA-derived smRCs were differentially expressed between HCC and controls. In fact, 250 smRCs were enough to distinguish them (online supplemental figure S5A). This led us to hypothesise that smRCs could be useful tools for early HCC detection. We selected the three top differentially expressed and low-complexity smRCs for further biomarker analysis (see online supplemental methods) and confirmed differential

expression between HCC and controls at risk (online supplemental figure 5B). Additional analysis in a cohort of 142 patients with non-HCC malignancies (100 patients with colon cancer, 6 patients with pancreatic adenocarcinoma and 36 patients with prostate cancer) further confirmed their HCC specificity (online supplemental figure 5B). We orthogonally validated the differential expression of our 3-smRC signature in this 'HCC biomarker discovery' cohort using RT-qPCR. Pearson's correlation coefficient was higher than 0.6 for all three smRCs when comparing data from small RNA sequencing and RT-qPCR ($n=15$, $p<0.05$, online supplemental figure S5C–E). The three smRCs were located in regions of chromosomes 3q, 8q (both unannotated intergenic regions) and 10q (intronic region of *SGPL1*) (online supplemental table S4). Altogether, smRCs are able to discriminate HCC and controls, including a 3-smRC signature that was orthogonally validated by RT-qPCR.

EV-associated smRCs are overexpressed in patients with early stage HCC compared with high-risk controls

To determine the clinical utility of smRCs in exRNA, we designed a phase 2 biomarker case-control study following the recommendations from the EDRN from the National Cancer Institute.¹⁶ In detail, we aimed at assessing the performance of our 3-smRC signature as a novel early detection biomarker in HCC. Unlike many studies in this setting,²⁴ we only enrolled patients with HCC at an early stage (BCLC classification stage 0 or A⁷), who can be cured with either surgery or ablation.⁷ Crucially, our control cohort is the target population for HCC surveillance as defined in clinical practice guidelines^{10 11} and a recent white paper on biomarker development for HCC by the International Liver Cancer Association.²⁵ We included 209 patients: $n=105$ treatment-naïve, early stage HCC, $n=85$ controls with cirrhosis

and/or CLD at high risk for HCC enrolled in HCC surveillance (table 1), and $n=19$ individuals without CLD (non-CLD). Our main matching criteria was the prevalence of cirrhosis as we believe this was potentially the strongest variable that could affect the performance of our biomarker. By comparing HCC and CLD groups, we did not observe clinically significant differences in the prevalence of cirrhosis, variables associated with liver function (bilirubin and albumin) or aetiology. As expected, HCC cases were slightly older and predominantly male gender compared with CLD (table 1). However, age and gender did not impact smRC expression (online supplemental figure S6A,B). We confirmed significant overexpression of our 3-smRC signature in plasma of patients with early stage HCC compared with CLD controls with RT-qPCR ($p<3e-5$, figure 4A, see online supplemental figure S6C for comparison with patients with non-CLD). To confirm the reproducibility of our biomarker analysis, we repeated the quantification of our 3-smRC early detection signature in 42 patients. This included EV enrichment from plasma, RNA extraction and RT-qPCR. These two independent experiments yielded a correlation coefficient of 0.83 ($p<0.001$, figure 4B). Longitudinal analysis in a subset of 30 patients with available sequential blood samples before and after HCC treatment revealed that smRC expression dynamics correlate with tumour response in these patients. In patients without early tumour recurrence after resection ($n=13$), smRC expression levels significantly decreased compared with baseline (paired t-test, figure 4C). Additional experiments showed significantly higher expression of smRC-48,615 in EV-enriched isolates as opposed to EV-depleted plasma ($n=30$ patients, figure 4D), suggesting the smRC signal is in fact EV associated.

In summary, we report on three smRCs with differential expression between early stage HCC and controls at high risk, who represent the target population for surveillance programmes, including replicates and longitudinal samples before and after HCC treatment.

Table 1 Clinical characteristics

	CLD, n=85*	HCC, n=105*	P value†
Age			0.15
≤60 years	38 (45%)	34 (33%)	
>60 years	47 (55%)	68 (67%)	
Gender			<0.001
Female	38 (45%)	20 (20%)	
Male	47 (55%)	82 (80%)	
Cirrhosis (Yes)	61 (72%)	68 (67%)	0.6
Bilirubin			0.5
≤1.2 mg/dL	55 (67%)	62 (73%)	
>1.2 mg/dL	27 (33%)	23 (27%)	
Albumin			0.10
≥3.5 g/dL	62 (77%)	54 (64%)	
<3.5 g/dL	19 (23%)	31 (36%)	
Aetiology			0.4
Non-viral	17 (31%)	40 (39%)	
Viral	37 (69%)	62 (61%)	
Tumour stage (BCLC)			
Very early (stage 0)	n.a.	22 (21%)	
Early (stage A)	n.a.	83 (79%)	
Single Nodule	n.a.	92 (90%)	
Largest nodule (cm)	n.a.	2.9 (2.0, 4.6)	
AFP (ng/mL)‡	4 (2, 5)	8 (4, 92)	<0.001

*Statistics presented: median (IQR); n (%).

†Statistical tests performed: Wilcoxon rank-sum test; χ^2 test of independence.

‡Upper limit of normal 9 ng/mL.

AFP, alpha-fetoprotein; BCLC, Barcelona Clinic for Liver Cancer; n.a., not applicable.

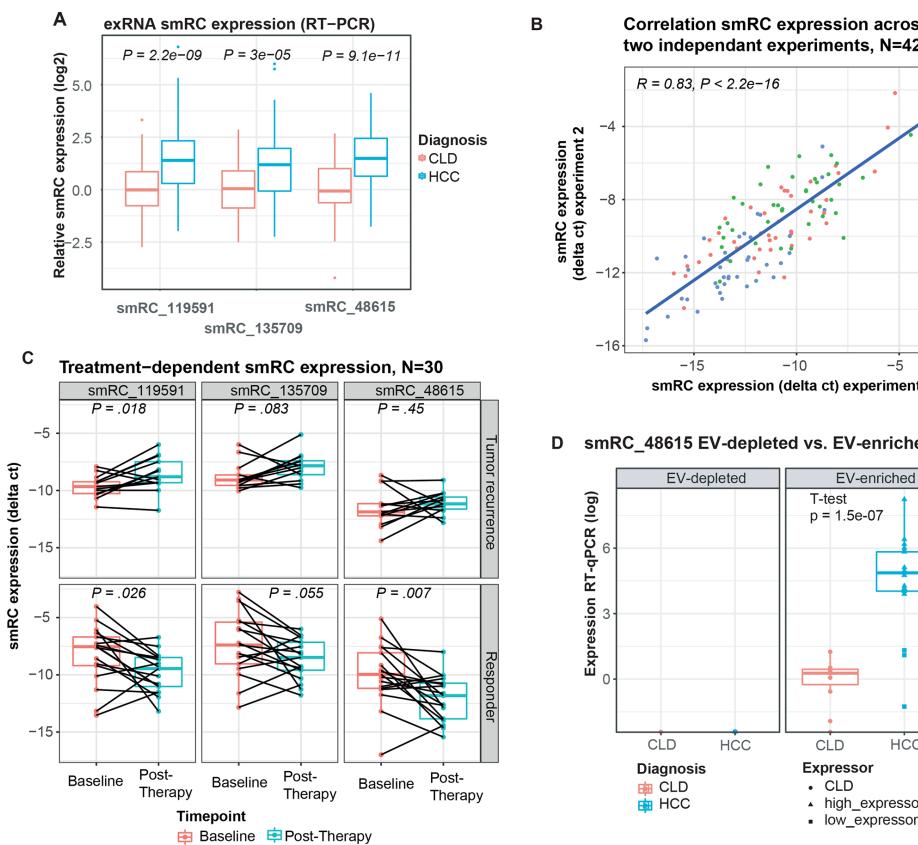


Figure 4 SmRC expression in 'HCC biomarker validation' cohort. (A) Expression for each smRC between patients with HCC and chronic liver disease (CLD) controls (centre line, median; box limits, upper and lower quartiles; whiskers, $1.5 \times$ IQR; points, outliers). (B) Correlation of biomarker analysis for all three smRCs in 42 patients across two independent experiments, including EV enrichment from plasma, exRNA extraction and RT-qPCR. (C) Longitudinal analysis of smRC expression in 30 patients with available sequential blood samples before and after HCC treatment (responders n=13, tumour recurrence n=17, paired t-test). Displayed is the smRC expression as delta between ct values of the spike-in control and respective smRC; smaller delta equals higher expression of the smRC. (D) Expression of smRC-48,615 in EV-enriched isolates and EV-depleted plasma. Displayed are samples from HCC and CLD controls. Triangles indicate HCC samples with relatively high expression and rectangles indicate samples with lower expression. CLD, chronic liver disease; ct, cycle threshold; EV, extracellular vesicle; exRNA, extracellular RNA; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription quantitative PCR; smRCs, small RNA clusters

specificity with a positive predictive value (ie, true positive rate) of 89% on average by maximising the balanced accuracy of the test ROC curves (online supplemental figure S7) and figure 6A,C. The AUC for our 3-smRC model was 0.87. Finally, a likelihood ratio test between an AFP-only early HCC detection model and one incorporating both AFP and our 3-smRC early detection signature showed that our smRCs add significant predictive power to AFP alone ($p < 0.0001$). As expected, AFP levels and expression of our 3-smRC signatures were not correlated (figure 6B), which suggest that both capture complementary signals for early HCC detection. Indeed, a blood-based composite model of our 3-smRC signature and AFP yielded an increased AUC of 0.93, lower B of 0.11 and better test performance (85% sensitivity, 94% specificity and positive predictive value of 95%; figure 6A,B, online supplemental table S2). These data confirm that our plasma 3-smRC signature robustly yields high accuracy in predicting early stage HCC among patients at high risk, independent of AFP. A composite model, including our 3-smRC signature and AFP, further enhances its performance.

DISCUSSION

Our study provides a conceptually novel solution to a key barrier in the field of exRNA-derived cancer biomarkers. We strongly depart from previous exRNA characterisation studies, which are

restricted to quantifying expression of known (ie, annotated) transcripts.^{13 14} Thus, we do not discard the substantial component of unannotated exRNA, or simply focus on a particular RNA biotype (eg, miRNA).^{27 28} Instead, we provide a novel, scalable and data-driven view of the entire small exRNA landscape unfettered by incomplete and emerging prior knowledge. This approach allowed us to identify and validate novel circulating biomarkers for the detection of curable, early stage HCC.

By de novo characterising the unknown non-coding small exRNA landscape across EV isolation technologies, biofluid and cancer type, we have defined the key properties of exRNA-associated smRCs, including their clinical application in early cancer detection. We have used a comprehensive dataset from prostate cancer patients to establish an exRNA-specific smRC feature set from which we mine their key statistical properties and develop selection criteria. These properties indicate that the tractable smRC-based quantification of novel, unannotated, small RNA expression signatures is feasible across different EV isolation techniques applied to different biofluids, potentially offering a completely novel, data-driven strategy for increasing the sensitivity of EV-associated biomarker discovery. It is important to emphasise that multiple small functional non-coding RNA can arise from transcriptional post-processing of a single larger RNA precursor gene (eg, endogenous small

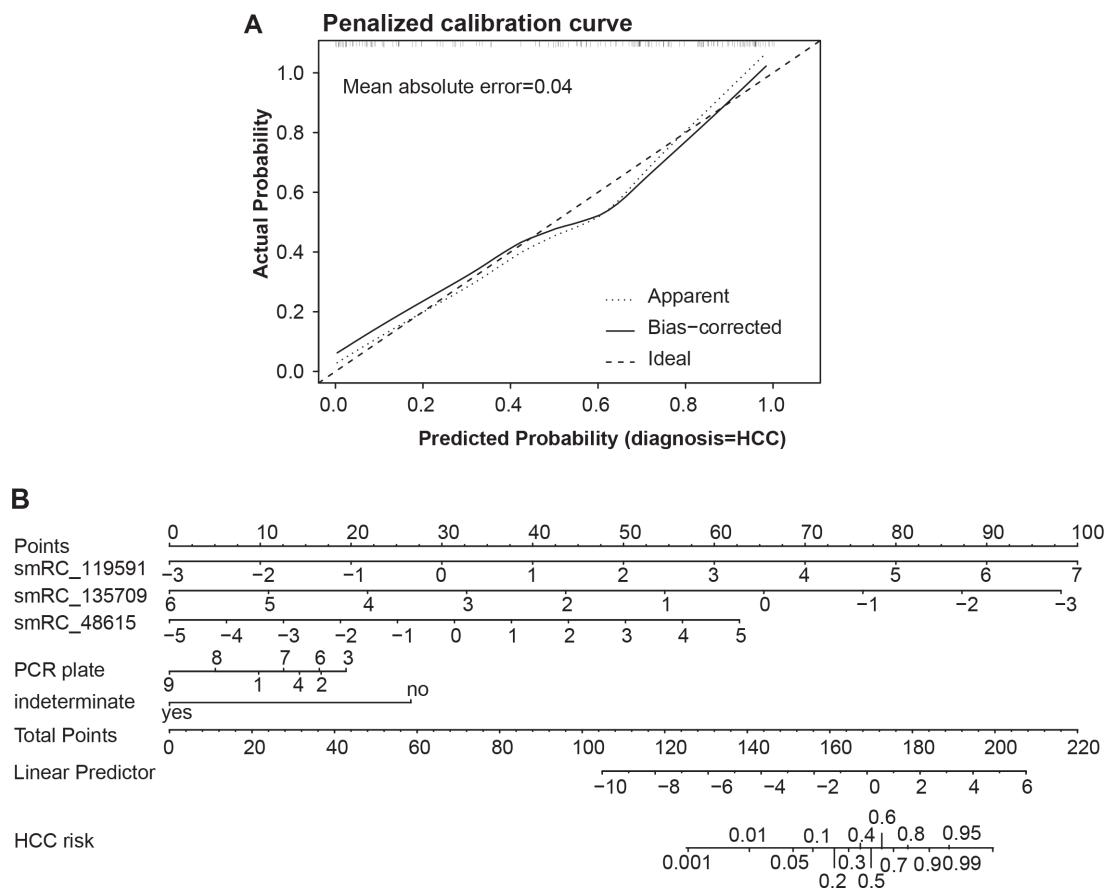


Figure 5 (A) Calibration curve for penalised smRC logistic regression model to predict early HCC, with mean error 0.04. (B) Nomogram for 3-smRC signature to predict early stage HCC. HCC, hepatocellular carcinoma; smRC, small RNA cluster.

interfering RNA (siRNAs) of plants²⁹ and animals,³⁰ miRNA hairpins yielding miRNA*³¹ and Piwi-interacting (piRNAs³²), so smRCs estimate the overlooked underlying expression profile of small RNA precursor genes and thereby facilitate accurate quantification, differential expression and motif discovery of unknown, heterogeneous, small RNA dominated exRNA payloads. In this sense, smRCs might more accurately measure the information content of exRNA.

Applying our approach to a separate HCC plasma-based exRNA dataset ($n=157$), we derived a 3-smRC (unannotated), HCC-specific signature, which was then validated in an independent HCC cohort ('HCC biomarker validation cohort', $n=209$) to discriminate patients with incipient HCC from controls at high risk of cancer. Despite the significant Wilcoxon p values demonstrating non-random separation of groups, the variability of smRC expression across HCC, and in fact CLD, was our primary motivation to turn to the more appropriate analytic setting of a full logistic regression model, which cannot only leverage the combined predictive power of even partially correlated smRC biomarker candidates, but can also effectively regress out technical bias (eg, sequencing batch plate effects from RT-qPCR). Within this context, extra-sample error can be robustly estimated using standard bootstrap resampling techniques, leading directly to robust estimates of calibration and prediction error and power (figure 5A) that accounts for overfitting. Generalisability of our results will be improved by external cohorts, ideally in a prospective setting, which are limitations of our study. Nevertheless, bootstrap resampling with replacement and cross-validation with a 85/15 test/training split both gave similar estimates of model optimism and extra-sample

generalisability when averaged over 1000 iterations, which have previously been shown to be robust predictors of extra-sample performance.³³ Guided by recommendations of the International Society of Extracellular Vesicles,¹⁹ a thorough characterisation of our isolates suggested a predominant enrichment for small EVs as a likely origin of our smRC signal. However, we cannot rule out contamination of other nanoparticles and we acknowledge technology-based differences for morphology assessment of EV isolates between NTA and TEM, particularly for size estimation, as reported previously.³⁴

Importantly, our exRNA-derived smRC signature was developed as a method for early HCC detection in the context of cancer surveillance and not as a HCC diagnostic tool. There is a subtle but very crucial difference between these two clinical scenarios, which directly determined the patient population we deliberately selected for this study, as extensively outlined in clinical guidelines^{10 11} and a recent white paper on HCC biomarker development.²⁵ Briefly, they explicitly underscore the urgent clinical need for new tools to detect patients with early stage HCC, as they can be cured if diagnosed at this stage. Other malignant liver tumours (eg, cholangiocarcinoma) and associated metastases rarely occur in patients with cirrhosis and are not the target of liver cancer surveillance programmes.⁷ Nevertheless, we have confirmed the HCC specificity of our 3-smRC signature in a dataset of 142 patients with other malignancies. We purposely chose to test our early detection biomarker candidates in the context of the hardest possible scenario of distinguishing between CLD and very early, curable, HCC. Our signature is independently validated in more than 200 patients, where we demonstrate its ability to accurately detect patients

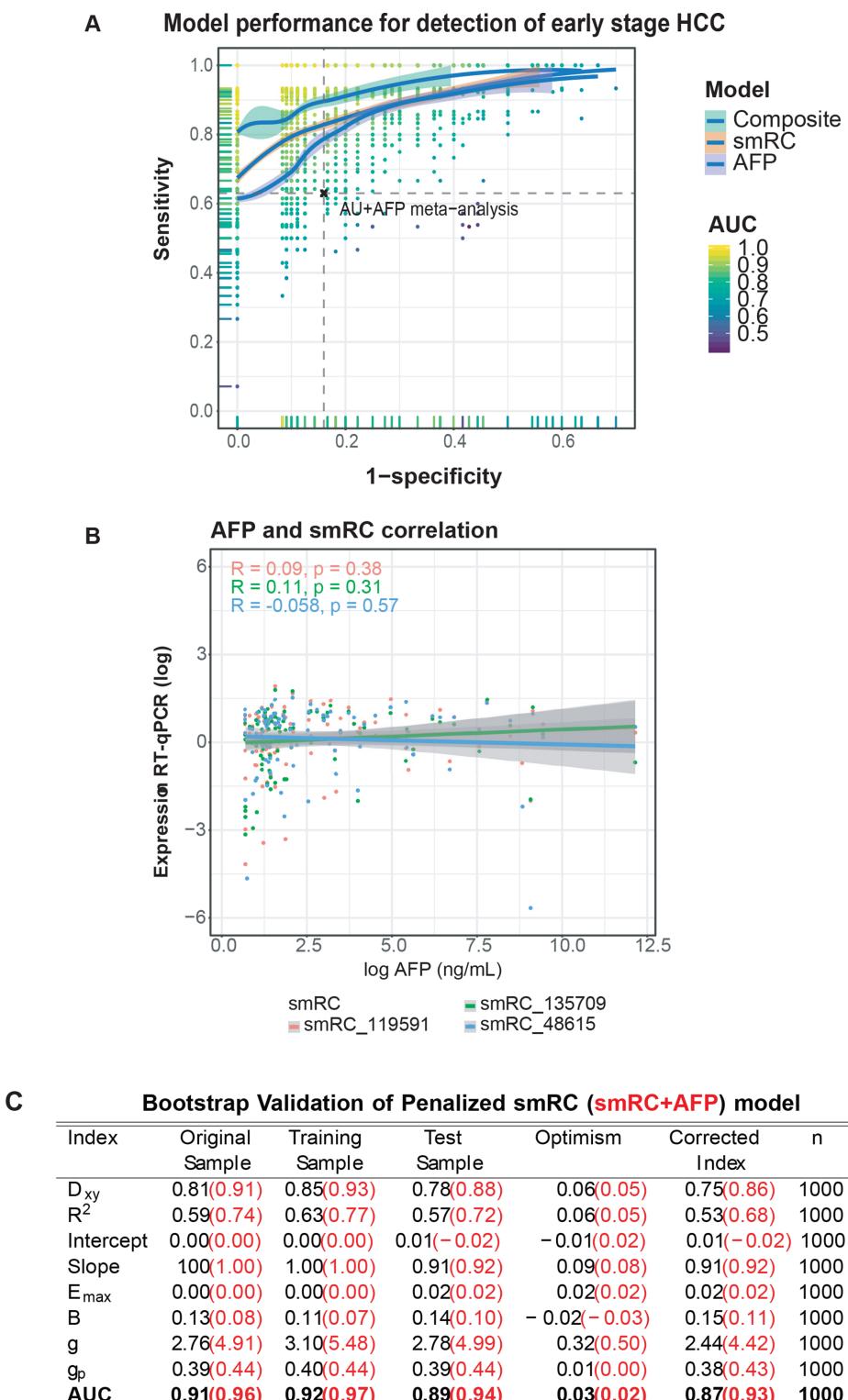


Figure 6 Performance of 3-smRC signature in a phase 2 biomarker case-control study. (A) ROC curve for maximised gain-of-certainty across repeated cross-validation. Each point represents a pair of sensitivities and specificities that maximise gain-in-certainty (ie, sensitivity+specificity) from a test validation ROC curve, whose AUC colours the point. The loess curves trace the best density fit of points across this space, with 95% CIs shown in grey. (B) AFP and smRC correlation plot. (C) Bootstrap validation parameters for smRC and smRC+AFP model. AFP, alpha-fetoprotein; AUC: area under the receiver operating curve; B: Brier score; CLD, chronic liver disease; D_{xy}: Somers' rank correlation between the observed HCC status and predicted HCC probabilities; E_{max}: maximum absolute calibration error on probability scale; g: Gini's mean difference of log-odds between HCC and CLD; g_p: Gini's mean difference in probability scale; HCC, hepatocellular carcinoma; ROC: receiver operating curve; RT-qPCR, reverse transcription quantitative PCR; smRC, small RNA cluster.

with early stage HCC, including technical replicates and longitudinal samples before and after HCC treatment. We demonstrate that our 3-smRC signature (86% sensitivity and 91%

specificity) not only outperforms the recommended surveillance tools (serum AFP combined with abdominal ultrasound: 63% sensitivity, 83% specificity)¹² for early stage HCC detection,

but is also complementary to AFP and in combination further maximises HCC detection rates. There are other approaches currently under evaluation for early HCC detection using other liquid biopsy analytes,³⁵ mostly involving circulating DNA. Blood-based DNA mutation,³⁶ methylation^{24,37} and DNA end-motif profiling³⁸ studies have shown comparable performance to our 3-smRC signature. The main difference with our study is that most of them included patients with HCC at more advanced stages³⁹ as opposed to our exclusively early stage cohort, and/or healthy controls, which might bias the performance of the tests.

Our study is a phase 2 biomarker case-control study according to the white paper by Pepe *et al* on phases of biomarker development¹⁶ and the EDRN guidelines by the National Cancer Institute. This design includes the use of unannotated small RNA sequences with the aim of assessing the performance of our three markers to discriminate early stage HCC and controls at high risk by inferring sensitivity and specificity of our test. Additionally, we wanted to put our results into clinical context. Therefore, we powered our study to compare against the performance of ultrasound and AFP for the detection of early stage HCC according to the largest meta-analysis available. Subsequent studies directly comparing our 3-smRC signature against ultrasound and AFP in a prospective setting will follow (ie, phase 3/4 biomarker studies).

Despite not yet having a clear functional role in oncogenesis apart from suggestive enrichments in key RNA binding protein motifs (online supplemental figure S8), our findings strongly suggest that unannotated smRCs enable a robust, blood-based, minimally invasive, operator-independent surveillance test for HCC, which is a major unmet clinical need in at-risk patients. In our study, a 3-smRC signature was able to detect HCC at an early tumour stage allowing patients to receive curative therapies, offering the potential to generalise this strategy to other cancer types as well. While further validation in phase 3/4 biomarker studies will pave the way for its clinical implementation, this study highlights complex, heterogeneous, non-coding and unannotated small RNA payloads of exRNA and their emergence as a powerful modality for biomarker discovery in cancer.

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Contributors Study design and drafting of the manuscript: JvF, GS, BL and AV. Sample collection: JvF, TG-L, ND, AC, IL, PH, DD'A, BS, PT, AA, DD, CC-C, AT, MS and AV. Experimental procedures: JvF, TG-L, ND, AC, SG, BW, JTS, SK, JELD, RO, DH, KA, RS, GS, BL and AV. Data analysis: JvF, TG-L, ND, EG-K, MEA, JELD, XC, PR, GH-M, JL, GS, BL and AV. All authors have critically revised the manuscript and gave their final approval.

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