Novel significant stage-specific differentially expressed genes in hepatocellular carcinoma

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Abstract

Background: Liver cancer is among top deadly cancers worldwide with a very poor prognosis, and the liver is a vulnerable site for metastases of other cancers. Early diagnosis is crucial for treatment of the predominant liver cancers, namely hepatocellular carcinoma (HCC). Here we developed a novel computational framework for the stage-specific analysis of HCC.

Methods: Using publicly available clinical and RNA-Seq data of cancer samples and controls and the AJCC staging system, we performed a linear modelling analysis of gene expression across all stages and found significant genome-wide changes in the log fold-change of gene expression in cancer samples relative to control. To identify genes that were stage-specific controlling for confounding differential expression in other stages, we developed a set of six pairwise contrasts between the stages and enforced a \( p \)-value threshold (< 0.05) for each such contrast. Genes were specific for a stage if they passed all the significance filters for that stage. The monotonicity of gene expression with cancer progression was analyzed with a linear model using the cancer stage as a numeric variable.

Results: Our analysis yielded two stage-I specific genes (CA9, WNT7B), two stage-II specific genes (APOBEC3B, FAM186A), ten stage-III specific genes including DLG5, PARI, NCAPG2, GNMT and XRCC2, and 35 stage-IV specific genes including GABRD, PGAM2, PECAM1 and CXCR2P1. Overexpression of DLG5 was found to be tumor-promoting contrary to the cancer literature on this gene. Further, GABRD was found to be significantly monotonically upregulated across stages. Our work has revealed 1977 genes with significant monotonic patterns of expression across cancer stages. NDUFA4L2, CRHBP and PIGU were top genes with monotonic changes of expression across cancer stages that could represent promising targets for therapy. Comparison with gene signatures from the BCLC staging system identified two genes, HSP90AB1 and ARHGAP42. Gene set enrichment analysis indicated overrepresented pathways specific to each stage, notably viral infection pathways in HCC initiation.

Conclusions: Our study identified novel significant stage-specific differentially expressed genes which could enhance our understanding of the molecular determinants of hepatocellular carcinoma progression. Our findings could serve as biomarkers that potentially underpin diagnosis as well as pinpoint therapeutic targets.

Keywords: LIHC transcriptomics, HCC stages, Stage-specific biomarkers, Differentially expressed genes, Pairwise contrasts, Significance analysis, Linear modelling, Tumorigenesis, Cancer progression, Metastasis, Monotonic expression
Background
Liver cancer is the second most deadly cancer in terms of mortality rate, with a very poor prognosis [60]. It accounted for 9.1% of all cancer deaths, and 83% of the annual new estimated 782,000 liver cancer cases worldwide occur in developing countries [13]. Liver cancer showed the greatest increase in mortality in the last decade for both males (53%) and females (59%) [8]. Liver hepatocellular carcinoma (LIHC) or simply hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for nearly 85% of liver cancers. 78% of all reported cases of HCC were due to viral infections (53% Hepatitis B virus and 25% Hepatitis C virus) [38]. There are several non-viral causes of HCC as well, mainly aflatoxins and alcohol [10]. As shown in Fig. 1, all the factors converge to a common mechanism of genetic alterations that lead to the acquisition of cancer hallmarks [20] and the eventual emergence of a cancer cell [11]. Genetic alterations constitute the heart of the problem, and studying changes due to these genetic
alterations is paramount to understand HCC. Earlier gene expression studies using EST data detected differential expression in cancer tissue compared to non-cancerous liver and proposed the existence of genetic aberrations and changes in transcriptional regulation in HCC [58]. The Cancer Genome Atlas (TCGA) research network [41] have subtyped and identified many potential targets for HCC based on a comprehensive multi-omics analysis. An independent analysis of TCGA RNA-Seq data encompassing 12 cancer tissues has uncovered liver cancer-specific genes [37]. Zhang et al. [63] have performed mutation analysis of HCC, and Yang et al. [59] combined TCGA expression data and natural language processing techniques to identify cancer-specific markers.

The burden of disease and mortality rate are both inversely correlated with the cancer stage. The response rate to therapy is also inversely correlated with stage. To the best of our knowledge, there are no reported research in the literature that have dissected the stage-specific features of HCC. The cancer staging system is based on gross features of cancer anatomical penetration, and one such standard is the American Joint Committee on Cancer (AJCC) Tumor-Node-Metastasis (TNM) staging [2]. It is reasonable to hypothesize that the stage-specific gross changes are associated with signature molecular events, and try to probe such molecular bases of stage-wise progression of cancer. We had earlier published on stage-specific “hub driver” genes in colorectal cancer [36]. A stage-focussed analysis of colorectal cancer transcriptome data yielded negative results vis-a-vis the AJCC staging system [25].

**Methods**

**Data preprocessing**

Normalized and log₂-transformed Illumina HiSeq RNA-Seq gene expression data processed by the RSEM pipeline [29] were obtained from TCGA via the firebrowse.org portal [6]. The patient barcode (uuid) of each sample encoded in the variable called ‘Hybridization REF’ was parsed and used to annotate the controls and cancer samples (Fig. 2). To annotate the stage information of the cancer samples, we obtained the clinical information dataset for HCC from firebrowse.org (LIHC.Merge_...
Clinical.Level_1.2016012800.0.0.tar.gz) and merged the clinical data with the expression data by matching the “Hybridization REF” in the expression data with the aliquot barcode identifier in the clinical data. The stage information of each patient was encoded in the clinical variable “pathologic stage”. The pathologic stage is essentially the surgical stage, prior to any treatment received, determined with the tissue obtained at the time of surgery. This interpretation is reinforced in the TCGA HCC sample inclusion criteria as follows: “Surgical resection of biopsy biospecimens were collected from patients diagnosed with hepatocellular carcinoma (HCC), and had not received prior treatment for their disease (ablation, chemotherapy, or radiotherapy)” (The TCGA [41]). The availability of this unequivocal information enables the analysis of cancer stages. The substages (A, B, C) were collapsed into the parent stage, resulting in four stages of interest (I, II, III, IV). We retained a handful of other clinical variables pertaining to demographic features, namely age, sex, height, weight, and vital status. With this merged dataset, we filtered out genes that showed little change in expression across all samples (defined as $\sigma < 1$). Finally, we removed cancer samples from our analysis that were missing stage annotation (value ‘NA’ in the “pathologic stage”). The data preprocessing was done using R (www.r-project.org).

**Table 2** Contrast matrix for inter-stage contrasts. There are six possible pairwise contrasts between the stages that are essential to identifying stage-specific genes.

|        | Stage2-Stage1 | Stage3-Stage1 | Stage3-Stage2 | Stage4-Stage1 | Stage4-Stage2 | Stage4-Stage3 |
|--------|---------------|---------------|---------------|---------------|---------------|---------------|
| Control | 0             | 0             | 0             | 0             | 0             | 0             |
| Stage1  | -1            | -1            | 0             | -1            | 0             | 0             |
| Stage2  | 1             | 0             | -1            | 0             | -1            | 0             |
| Stage3  | 0             | 1             | 1             | 0             | 0             | -1            |
| Stage4  | 0             | 0             | 0             | 1             | 1             | 1             |
Linear modelling of expression across cancer stages relative to the baseline expression (i.e., in normal tissue controls) was performed for each gene using the R limma package [42]. The following linear model was fit for each gene's expression based on the design matrix shown in Fig. 3a:

\[ y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 \]  

where the independent variables are indicator variables of the sample's stage, the intercept \( \alpha \) is the baseline expression estimated from the controls, and \( \beta_i \) are the estimated stage-wise log fold-change (lfc) coefficients relative to controls. The linear model was subjected to empirical Bayes adjustment to obtain moderated t-statistics [34]. To account for multiple hypothesis testing and the false discovery rate, the \( p \)-values of the F-statistic of the linear fit were adjusted using the method of Hochberg and Benjamini [22]. The linear trend across cancer stages for the top significant genes were visualized using boxplots to ascertain the regulation status of the gene relative to the control.

Monotonic mean expression

The linear model in eqn. (1) would not be sufficient to identify genes with an ordered monotonic trend of expression across cancer stages. Addressing this question would also help assess whether monotonic changes of gene expression were observed with disease progression. Towards this end, we designed a model of gene expression where the cancer stage was treated as a numeric variable:

\[ y = aX + b \]  

where \( X \) takes a value in \[0,1,2,3,4\] corresponding to the sample stage: [control, I, II, III, IV], respectively. It

| TCGA Stage | TNM classification | Cases |
|------------|--------------------|-------|
| 1A         | T1a N0 M0          | 172   |
| 1B         | T1b N0 M0          |       |
| 2          | T2 N0 M0           | 87    |
| 3A         | T3 N0 M0           | 65    |
| 3B         | T4 N0 M0           | 8     |
| 3C         | -                  | 9     | 85   |
| 3          | -                  | 3     |
| 4A         | T(any) N1 M0       | 1     | 5    |
| 4B         | T(any) N(any) M1   | 2     |
| 4          | -                  | 2     |
| CONTROL    | -                  | 50    |
| NA         | -                  | 24    |
Table 4  Summary of key demographic features of the dataset. For continuous variables (age, height, weight and BMI), the mean ± standard deviation is given. BMI is calculated only for patients with both height and weight data

| Characteristic | Control | Stage1 | Stage2 | Stage3 | Stage4 | NA | Overall |
|---------------|---------|--------|--------|--------|--------|----|---------|
| Number of samples | 50      | 172    | 87     | 85     | 5      | 24 | 423     |
| Age (Years)    | 61.7±16.1 | 60.6±12.2 | 59.0±13.3 | 56.2±14.8 | 42.8±20.7 | 68.1±10.7 | 59.7±13.2 |
| Height (cm)    | 170.6±9.5  | 166.5±12.3 | 167.9±8.3  | 169.0±8.9  | 162.3±4.9  | 166.2±11.1 | 167.7±10.6 |
| Weight (Kg)    | 76.1±22.1  | 73.2±19.8  | 73.3±18.9  | 69.9±18.8  | 72.3±21.5  | 79.7±19.8  | 73.2±19.7  |
| BMI            | 26.2±7.8  | 26.7±10.2  | 26.0±5.8  | 24.3±6.0  | 27.7±9.3  | 29.1±7.6  | 26.1±8.4  |
| Sex            | Male     | 28      | 122     | 60      | 55      | 1   | 14      | 280     |
|                | Female   | 22      | 50      | 27      | 30      | 4   | 10      | 143     |
| Vital Status   | Alive    | 20      | 134     | 71      | 65      | 2   | 12      | 304     |
|                | Dead     | 30      | 38      | 16      | 20      | 3   | 12      | 119     |

Table 5  Top 10 genes of the linear model. The log-fold change expression of the gene in each stage relative to the controls are given, followed by p-value adjusted for the false discovery rate, and the regulation status of the gene in the cancer stages with respect to the control

| Genes    | Stage I Lfc ($\beta_1$) | Stage II Lfc ($\beta_2$) | Stage III Lfc ($\beta_3$) | Stage IV Lfc ($\beta_4$) | Adj. p value | Regulation status |
|----------|--------------------------|--------------------------|--------------------------|--------------------------|--------------|-------------------|
| GABRD    | 5.08                     | 5.11                     | 5.24                     | 6.55                     | 5.529e-78    | Up-regulated      |
| PLVAP    | 3.51                     | 3.24                     | 3.24                     | 3.79                     | 7.498e-75    | Up-regulated      |
| CLEC4M   | -8.32                    | -8.67                    | -8.48                    | -9.24                    | 6.058e-74    | Down-regulated    |
| CXORF36  | 2.91                     | 2.86                     | 2.76                     | 3.44                     | 5.376e-73    | Up-regulated      |
| CLEC1B   | -7.85                    | -8.46                    | -8.05                    | -9.44                    | 6.292e-71    | Down-regulated    |
| BMP10    | -4.66                    | -4.75                    | -4.67                    | -5.25                    | 1.447e-66    | Down-regulated    |
| CLEC4G   | -7.75                    | -8.23                    | -7.95                    | -8.75                    | 2.437e-66    | Down-regulated    |
| CDH13    | 3.30                     | 3.34                     | 3.32                     | 3.86                     | 3.454e-66    | Up-regulated      |
| UBE2T    | 3.85                     | 4.50                     | 4.47                     | 3.76                     | 2.544e-65    | Up-regulated      |
| SLC26A6  | 3.10                     | 3.39                     | 3.34                     | 3.07                     | 7.438e-65    | Up-regulated      |
was noted the mean expression of a gene could show the following monotonic patterns across cancer stages:

(i). monotonic upregulation, where mean expression follows: control < I < II < III < IV.
(ii). monotonic downregulation, where mean expression follows: control > I > II > III > IV.

The sets of genes conforming to either (i) or (ii) were identified to yield monotonically upregulated and monotonically downregulated genes. These two sets were merged, and the final set of genes with monotonic changes of expression with cancer progression was obtained. This final set was ranked by the adj. $p$-values from the model estimated by eqn. (2).

**Pairwise contrasts**

To perform contrasts, a slightly modified design matrix shown in Fig. 3b was used, which would give rise to the following linear model of expression for each gene:

$$ y = \beta_0 x_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 $$

where the controls themselves are one of the indicator variables, and the $\beta_i$ are all coefficients estimated only from the corresponding samples. Our first contrast of interest, between each stage and the control, was achieved using the contrast matrix shown in Table 1. Four contrasts were obtained, one for each stage vs control. A threshold of $|\text{lfc}| > 2$ was applied to each such contrast to identify differentially expressed genes (with respect to the control). We used the absolute value of the lfc, since driver genes could be either upregulated or downregulated. Genes could be differentially expressed in any combination of the stages or no stage at all. To analyze the pattern of differential expression (with respect to the control), we constructed a four-bit binary string for each gene, where each bit signified whether the gene was differentially expressed in the corresponding stage. For example, the string ‘1100’ indicates that the gene was differentially expressed in the first and second stages. There are $2^4 = 16$ possible outcomes of the four-bit string for a given gene corresponding to different patterns of expression.
to the combination of stages in which it is differentially expressed. This is illustrated in set-theoretic terms in Fig. 4.

In our first elimination, we removed genes whose $|\text{lfc}| < 2$ for all stages. For each remaining gene, we identified the stage that showed the highest $|\text{lfc}|$ and assigned the gene as specific to that stage for the rest of our analysis.

**Significance analysis**

We applied a four-pronged criteria to establish the significance of the stage-specific differentially expressed genes.

(i). Adj. $p$-value of the contrast with respect to the control $< 0.001$. The expression profile of a driver gene in cancer samples would markedly depart from that for the controls, which motivates the use of a stringent threshold here.

(ii). (ii)-(iv) $p$-value of the contrast with respect to other stages $< 0.05$. The use of a more relaxed cutoff would improve the sensitivity of stage-specific detection.

To obtain the above $p$-values (ii) - (iv), we used the contrast matrix shown in Table 2, which was then used an an argument to the contrastsFit function in limma.

**Further analyses**

Principal component analysis (PCA) were performed using prcomp in R. To choose 100 random genes, we used the rand function. Gene set enrichment analysis were performed on KEGG (https://www.genome.jp/kegg/) and Gene Ontology [5] using kegg and goana in limma, respectively. In order to visualize outlier genes that are significant with a large effect size, volcano plots could be obtained by plotting the -log10 transformed $p$-value vs. the log fold-change of gene expression. Heat maps of significant stage-specific differentially expressed genes were visualized using heatmap and clustered using hclust. Novelty of the identified stage-specific genes was ascertained by screening against the Cancer Gene Census v84 [14].

**Results**

The TCGA expression data consisted of expression values of 20,532 genes in 423 samples. After the completion of data pre-processing, we obtained a final dataset of expression data for 18,590 genes across 399 samples annotated with the corresponding sample stage (available in Supplementary File S1). The stagewise distribution of TCGA samples along with the corresponding AJCC staging is shown in Table 3. A statistical summary of demographic details including age, sex, height, weight, and vital status is shown in Table 4. The body mass index (BMI) distribution was derived from patient clinical data that had both height and weight (i.e, neither was ‘NA’). The average age of
onset of HCC was around 60 years, and the average BMI was about 26, indicating a possible link with age-related pathology and obesity.

The dataset was processed through voom in limma to prepare for linear modelling [28]. At a \( p \)-value cutoff of 0.05, 14,843 genes were significant for the linear model given by eqn. (1). Even raising the bar to 1E-5, 9618 genes remained significant in the linear modelling, thus implying a strong linear trend in their expression across cancer stages relative to control. This was not entirely surprising since one of the hallmarks of cancer phenotype is genome-wide instability [20]. The linear modelling highlighted top ranked genes, some upregulated in HCC (GABRD, PLVAP, CDH13) and some downregulated (CLEC4M, CLEC1B, CLEC4G). The lfc for each stage with respect to control of top ten genes (ranked by adjusted \( p \)-value) are shown in Table 5, along with their inferred regulation status. Boxplots of the expression of the top 9 genes (Fig. 5) indicated elevated expression across cancer stages relative to control for up-regulated genes, while depressed expression across cancer stages relative to control was indicative of downregulated genes. (Boxplots of all other genes in the top 200 are provided in the Supplementary Fig. S1) It is worthwhile to note that a given gene might have maximal differential expression in any stage (not necessarily stage 4), and the linear trend does not suggest the order of expression across stages (Fig. 6).

A PCA of the top 100 genes from the linear model was visualized using the top two principal components (Fig. 7a). A clear separation of the controls and the cancer samples could be seen, suggesting the extent of differential expression of these genes in cancer samples. Hence linear modelling yields cancer-specific genes versus normal controls, and the results for the all the genes, including the top 100, are provided in order in Supplementary File S3. The size of each such partition is illustrated in Fig. 8. We eliminated the 16,135 genes corresponding to the stage string ‘0000’ (lfc < 2 in all stages). To establish the significance of the remaining genes, we applied the second contrast (Table 3) and passed each gene through the four filter criteria. The gradual reduction in candidate stage-specific genes as each criterion was applied, is shown in Table 7. Only genes that passed all criteria were retained as significant stage-specific differentially expressed genes. We obtained 2 stage-I specific, 2 stage-II specific, 10 stage-III specific and 35 stage-IV specific genes (Table 8). Figure 9 shows the volcano plot of these 49 stage-specific genes.

In view of the limited sample size for stage-IV and consequent low power for rejecting false-positives, we stipulated that each stage-IV specific gene would display a smooth increasing or decreasing expression trend through cancer progression culminating in maximum differential expression in stage-IV. On this basis, we pruned the 35 stage-IV specific genes to just the top ten by significance in the linear modelling. This yielded a total of 24 stage-specific genes of interest.

A heatmap of the lfc expression of these stage-specific genes across the stages was generated (Fig. 10a) and revealed a systematic gradient in expression relative to control, involving both downregulation and overexpression. The map was clustered on the basis of differential expression (i.e., |lfc|) across stages and across features (i.e., genes) (Fig. 10b). It was seen that stage I genes clustered together, stage II genes co-clustered with NCAPG2 and DLG5 from stage-III, all the other stage-III genes clustered together, while the stage-IV genes formed two separate clusters. It was interesting to note that GABRD emerged as an outgroup to all the clusters, demonstrating its uniqueness.

To identify the biological processes specific to each stage, we used the genes with maximal |lfc| in each stage and performed a stagewise gene set enrichment analysis on two ontologies, the GO and KEGG pathways. Salient
results with respect to KEGG pathways are presented below (Table 9) and the complete KEGG and GO results are available in Supplementary Tables S1 and S2, respectively. In stage I, we found the significant enrichment of cell-cycle signaling pathways (Hippo, Wnt, HIF-1), and viral infection-related pathways (cytokine-cytokine receptor interaction, human papillomavirus infection, HTLV-1 infection). In stage II, key signalling pathways (Ras, MAPK) were aberrant. Two liver-specific pathways, alcoholism and cytochrome P450 mediated metabolism of xenobiotics were enriched, as well as standard cancer pathways of bladder, brain, stomach, and skin that might involve generic genetic alterations necessary for cancer cell growth. In stage III, we noticed the significant enrichment of Metabolic pathways that summarize cellular metabolism. This might indicate the metabolic shift needed by the cancer to grow and invade neighboring tissues. Other salient significantly enriched pathways pertained to increased cell cycle progression, DNA replication, chemical carcinogenesis, p53 signaling pathway and cellular senescence, all hallmark processes critical to cancer progression. Stage IV gene set was significantly enriched for bile-related processes (bile secretion, primary bile acid biosynthesis), and ABC transporters (possibly conferring a drug-resistant advanced cancer phenotype). A signaling pathway related to diabetic complications was enriched as well, indicating the role of co-morbidities in driving liver cancer progression. The enrichment analysis of the top 100 genes of the linear model is included in the Supplementary Table S3.

Discussion
When differentially expressed genes are identified in a two-class cancer vs control manner, the information about stage-specificity of differential expression is lost. By applying our protocol, this information is recovered and available for dissection. The top linear model genes and all the stage-specific differentially expressed genes (Table 10) were analyzed with respect to the existing literature.

Top genes of linear models
Three C-type lectin domain proteins (CLEC4M, CLEC1B, CLEC4G) were detected in the top ten genes of linear model given by eqn. (1). Interestingly, this identical cluster of three genes was detected as the most significantly downregulated liver cancer-specific genes in a qPCR study of an independent cohort of 65 tumor-normal matched cases [21]. On screening the top 200 linear model (1) genes against cancer driver genes in the Cancer Gene Census, only four genes were found, namely BUB1B, CDKN2A, EZH2, and RECQL4. The top 200 genes of the linear model given by eqn. (2) overlapped with 111 genes of linear model (1) and yielded six genes from the Cancer Gene Census, namely BUB1B, EZH2, CDKN2C, CANT1, POLD1, and STIL. Both CDKN2A and CDKN2C are cyclin-dependent kinase inhibitors. CDKN2A was a member of the gene signatures for HCC prognosis independently proposed by Gillet et al. [16] and Yang et al. [59]. It was remarkable that GABRD stood out as the top gene in both the linear models, and with a monotonic order of expression with the cancer stage. GABRD is discussed further in the section on Stage-IV specific genes. A gene with a monotonicity of expression may be increasingly upregulated as the cancer initiates, progresses and metastasizes, signalling its oncogenic progression; or conversely, it may be increasingly downregulated with the cancer stages, signalling the loss of tumor suppressor activity. Screening the top 200 genes with monotonic expression against
the Cancer Gene Census yielded a completely different set of six genes: HSP90AB1, ALDH2, ESR1, PPP2R1A, HIST1H4I, SEPT5. HSP90AB1, a heat shock protein and molecular chaperone, was a key result of Xu et al. [56] where it played a dual role, one in the set of 50 hub genes correlated with Barcelona Clinic Liver Cancer (BCLC) staging of HCC patients, and another, in the set of 13 hub genes correlated with overall survival of HCC patients. HSP90AB1 might have a significant role in the aetiology of HCC, given that its expression is known to be upregulated by hepatitis B virus encoded X protein [31]. The monotonic changes in HSP90AB1 might further facilitate its known roles in angiogenesis [19].

Table 6 Top 20 genes with significant monotonic patterns of expression. Intercept, Coefficient and Adj. p-value are from the linear model given by eqn.(2). Status indicates monotonic upregulation (UP) or monotonic downregulation (DOWN). The genes are sorted by significance (adj.p-value)

| Gene       | Intercept | Coefficient | Adj.p-value | status |
|------------|-----------|-------------|-------------|--------|
| GABRD      | -0.96     | 1.09        | 2.33E-023   | UP     |
| PIGU       | 3.80      | 0.43        | 2.45E-023   | UP     |
| NDUFA4L2   | 2.42      | 0.80        | 3.09E-023   | UP     |
| CRHBp      | 2.59      | -1.52       | 3.14E-023   | DOWN   |
| C20orf20   | 3.26      | 0.39        | 4.84E-023   | UP     |
| PPIA       | 7.00      | 0.34        | 1.65E-022   | UP     |
| C12orf47   | 2.31      | 0.39        | 2.75E-022   | UP     |
| VIPR1      | 2.40      | -1.12       | 4.60E-022   | DOWN   |
| FLVCR1     | 0.73      | 0.72        | 5.12E-022   | UP     |
| TTC13      | 2.73      | 0.50        | 1.06E-021   | UP     |
| NXPH4      | -2.29     | 1.31        | 3.53E-021   | UP     |
| CACYBP     | 4.87      | 0.42        | 4.65E-021   | UP     |
| MFSD5      | 3.98      | 0.34        | 5.15E-021   | UP     |
| PIGC       | 4.08      | 0.40        | 5.90E-021   | UP     |
| ATP6AP1    | 6.40      | 0.33        | 6.52E-021   | UP     |
| DYNLL1     | 6.37      | 0.32        | 7.09E-021   | UP     |
| CCT4       | 6.28      | 0.30        | 1.02E-020   | UP     |
| SLC41A3    | 4.29      | 0.36        | 1.11E-020   | UP     |
| ZBTB9      | 2.31      | 0.38        | 1.17E-020   | UP     |
| NEU1       | 5.92      | 0.43        | 1.20E-020   | UP     |

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Our study provides a more quantitative account of the significant monotonic downregulation of CRHBP with the HCC stage. Two proteins of the glycosylphosphatidylinositol (GPI) anchoring system, PIGU and PIGC, were top genes with respect to significant monotonic expression (Table 6); of these, PIGU is a known bladder cancer oncogene [18].

Stage-I specific DEGs (Fig. 12)
CA9 is a member of carbonic anhydrases, which are a large family of zinc metalloenzymes that catalyse the reversible hydration of carbon dioxide. Its expression in clear cell Renal carcinoma, but not in functional kidney cells has gained attention for its use as a pre-operative biomarker [30]. The WNT7B protein is part of the Wnt family, a family of secreted signalling proteins. Elevated WNT7B in pancreatic adenocarcinoma has been found to mediate anchorage independent growth [4]. Surprisingly, both CA9 and WNT7B are downregulated in HCC, most so in stage-I, contrary to their role in other cancers. A concrete interpretation of the role of these genes in HCC awaits appropriately designed experimental studies.

It is pertinent to ask the following question here: which genes are essential for the initiation of HCC?

### Table 7
Number of genes in each step of the significance analysis. Differential expression is defined with respect to a threshold $|\log FC| = 2$. Significance analysis proceeds first by significance (i.e. p-value) with respect to control, followed by p-value in each possible pairwise contrast between the different stages. Exclusive DE genes refer to genes differentially expressed in only one of the four stages (corresponding to the bit strings ‘1000’, ‘0100’, ‘0010’ and ‘0001’).

| Filtering criteria | STAGE 1 | STAGE 2 | STAGE 3 | STAGE 4 | Total |
|--------------------|---------|---------|---------|---------|-------|
| Exclusive DE genes | 40      | 75      | 223     | 481     | 819   |
| DE genes           | 122     | 407     | 844     | 1082    | 2455  |
| Adj.p-value w.r.to control | 120 | 406 | 839 | 293 | 1658 |
| p-value 1 x 2      | 26      | 187     | -       | -       | 213   |
| p-value 1 x 3      | 19      | -       | 670     | -       | 689   |
| p-value 1 x 4      | 2       | -       | -       | 88      | 90    |
| p-value 2 x 3      | -       | 13      | 70      | -       | 83    |
| p-value 2 x 4      | -       | 2       | -       | 46      | 48    |
| p-value 3 x 4      | -       | -       | 10      | 35      | 45    |
| Final genes        | 2       | 2       | 10      | 35      | 45    |
Clearly these genes would be differentially expressed in stage I relative to control. All significantly differentially expressed genes with maximal \( |\text{lfc}| \) in stage-I would be the best candidates for genes involved in the initiation of HCC. These 122 genes are provided in the Supplementary File S3.

| STAGE 1   | STAGE 2   | STAGE 3   | STAGE 4   |
|-----------|-----------|-----------|-----------|
| CA9       | FAM186A   | C12orf48  | GABRD     |
| WNT7B     | APOBEC3B  | C15orf42  | PECAM1    |
|           |           | ORC6L     | LOC25845  |
|           |           | ECT2      | CEND1     |
|           |           | WDHD1     | GBX2      |
|           |           | DLG5      | PGAM2     |
|           |           | XRCC2     | NR1I2     |
|           |           | NCAPG2    | GDF5      |
|           |           | GNMT      | CXCR2P1   |
|           |           | PRR11     | GPR1      |
|           |           |           | MUSTN1    |
|           |           |           | EHD2      |
|           |           |           | LOC143188 |
|           |           |           | HIST3H2BB |
|           |           |           | CA12      |
|           |           |           | CDX1      |
|           |           |           | MYO16     |
|           |           |           | CPE       |
|           |           |           | LPRR3     |
|           |           |           | ZMYND12   |
|           |           |           | KCNF1     |
|           |           |           | GPR126    |
|           |           |           | MCD1      |
|           |           |           | GABRB2    |
|           |           |           | SNCB      |
|           |           |           | TRIM50    |
|           |           |           | MT3       |
|           |           |           | KCNO2     |
|           |           |           | DUXA      |
|           |           |           | C14orf72  |
|           |           |           | ECEL      |
|           |           |           | FOXE      |
|           |           |           | MYH13     |
|           |           |           | ARHGAP42  |
|           |           |           | BMP7      |

**Stage-II specific DEGs (Fig. 13)**

APOBEC3B, a DNA cytidine deaminase, is a known cancer driver gene in the Cancer Gene Census, but there are no literature reports of its stage-specificity in any cancer. It is known to account for half the mutational load in breast carcinoma, and its target sequence context
Fig. 9 Volcano plot of the 49 significant stage-specific differentially expressed genes. Stage 1 genes, red; Stage 2, blue; Stage 3, green; and Stage 4, orange. The genes are seen to orient away from the origin and the axes, indicating significance and effect size.

Fig. 10 Heatmap plots of the final 24 stage-specific genes. a heatmap generated from the lfc values of all the stage-specific genes (arranged stagewise). The color gradient spans the spectrum from downregulation (blue) to overexpression (red). Log fold changes up to sixfold are seen, indicating 64 times differential expression with respect to control. b Representation of the stagewise gene expression based on clustering of differential expression profiles.
was found to be highly mutated in Bladder, lung, cervix, neck, and head cancers as well [7]. Further studies have attributed specific hypermutation signatures across all cancers to the APOBEC family, including APOBEC3B [1]. Here APOBEC3B is upregulated, increasing its capacity to inflict the hypermutator phenotype, and highlighting an intriguing stage-specificity in its action.

FAM186A polymorphisms have been reported in GWAS and SNP studies on colorectal cancer patients and shown to have a significant odds ratio in risk heritability [48].

FAM163A was a component of the 8-gene signature used for the risk stratification of HCC patients [39].

### Stage-III specific DEGs (Fig. 14)

C12orf48, also known as PARI, participates in the homologous recombination pathway of DNA repair, and its overexpression has been reported in pancreatic cancer [35]. Further PARI was recently identified as a transcriptional target of FOXM1 [62], which is a well-validated upregulated gene in HCC [21]. DLG5 is a cell

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**Table 9** Gene set enrichment analysis. Stage-specific gene sets (all the differentially expressed genes, corresponding to row 'DE genes' in Table 6) were analyzed for significant enrichment with respect to KEGG Pathways. Significance was based on p-value <0.05

| Stage | Enriched pathways                                                      | p-value  |
|-------|------------------------------------------------------------------------|----------|
| Stage 1 | Hippo signalling pathway                                               | 3.276e-03|
|       | Cytokine-cytokine receptor interaction                                 | 1.218e-02|
|       | Wnt signalling pathway                                                 | 1.528e-02|
|       | Human papillomavirus infection                                         | 1.763e-02|
|       | HTLV-I infection                                                       | 2.552e-02|
|       | HIF-1 signalling pathway                                               | 2.787e-02|
| Stage 2 | Bladder cancer                                                         | 4.643e-03|
|       | Ras signalling pathway                                                 | 5.264e-03|
|       | Pathways in cancer                                                     | 6.211e-03|
|       | Glioma                                                                | 6.457e-03|
|       | Alcoholism                                                             | 1.027e-02|
|       | Gastric cancer                                                         | 1.210e-02|
|       | MAPK signalling pathway                                                | 2.526e-02|
|       | Melanoma                                                               | 3.183e-02|
|       | Metabolism of xenobiotics by cytochrome P450                            | 3.472e-02|
| Stage 3 | Cell cycle                                                             | 2.881e-18|
|       | DNA replication                                                        | 6.526e-11|
|       | Chemical carcinogenesis                                                | 1.233e-06|
|       | Metabolic pathways                                                     | 1.204e-03|
|       | Cellular senescence                                                    | 7.203e-03|
|       | p53 signalling pathway                                                 | 7.275e-03|
|       | Bile secretion                                                         | 2.479e-06|
| Stage 4 | ABC transporters                                                       | 7.146e-06|
|       | Primary bile acid biosynthesis                                         | 2.357e-03|
|       | AGE-RAGE signalling pathway in diabetic complications                  | 3.024e-02|

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Sarathi and Palaniappan *BMC Cancer* (2019) 19:663
Table 10 Stage-wise effect sizes and significance of stage specific genes. The stage-wise log fold changes of differential expression of each candidate stage-specific gene in tumor samples relative to normal control samples are shown, along with significance values, and its inferred regulation status. In stage-IV, only the top 10 genes are shown. The stage-specificity of the genes are emphasized.

| GENE      | $\beta_0$ | $\beta_1$ | $\beta_2$ | $\beta_3$ | $\beta_4$ | Adj.p.value (from contrasts against control) | Adj.p.value (from linear model) | Regulation status (Up/Down) |
|-----------|-----------|-----------|-----------|-----------|-----------|---------------------------------------------|--------------------------------|---------------------------|
| **STAGE 1**                                      |           |           |           |           |           |                              |                                |                           |
| CA9       | 0.1       | -2.5      | -0.9      | 0.2       | 1.3       | 3.66e-05                                    | 3.44e-09                        | Down                      |
| WNT7B     | -1.8      | -2.3      | -1.4      | -1.1      | 0.3       | 5.45e-07                                    | 2.07e-06                        | Down                      |
| **STAGE 2**                                      |           |           |           |           |           |                              |                                |                           |
| APOBEC3B  | -0.9      | 2.0       | 2.6       | 1.7       | -0.6      | 1.12e-10                                    | 1.17e-09                        | Up                        |
| FAM186A   | -4.2      | 1.7       | 2.2       | 1.8       | 0.4       | 2.62e-18                                    | 1.50e-12                        | Up                        |
| **STAGE 3**                                      |           |           |           |           |           |                              |                                |                           |
| NCAPG2    | 1.5       | 1.5       | 1.8       | **2.1**   | 0.4       | 6.03e-25                                    | 1.76e-24                        | Up                        |
| DLG5      | 2.3       | 1.8       | 2.1       | **2.5**   | 1.1       | 1.23e-29                                    | 2.90e-29                        | Up                        |
| GNMT      | 6.9       | -1.6      | -2.6      | **3.6**   | -1.3      | 9.69e-16                                    | 2.40e-15                        | Down                      |
| PRR11     | -3.8      | 2.1       | 2.7       | **3.4**   | 1.0       | 1.74e-14                                    | 4.65e-13                        | Up                        |
| WDHD1     | -1.4      | 2.3       | 2.7       | **3.2**   | 1.8       | 2.25e-31                                    | 8.87e-31                        | Up                        |
| XRCC2     | -3.8      | 2.9       | 3.2       | **3.8**   | 1.9       | 2.29e-28                                    | 7.06e-28                        | Up                        |
| C12orf48  | -1.9      | 2.8       | 3.2       | **3.7**   | 2.4       | 5.88e-43                                    | 1.19e-43                        | Up                        |
| C15orf42  | -3.5      | 3.7       | 4.2       | **4.7**   | 3.2       | 1.52e-41                                    | 1.17e-42                        | Up                        |
| ECT2      | 0.2       | 2.5       | 3.0       | **3.5**   | 2.2       | 2.62e-35                                    | 4.29e-35                        | Up                        |
| ORC6L     | -2.4      | 3.1       | 3.5       | **4.0**   | 2.6       | 4.19e-41                                    | 5.55e-42                        | Up                        |
| **STAGE 4**                                      |           |           |           |           |           |                              |                                |                           |
| GABRD     | -3.8      | 5.1       | 5.1       | 5.2       | **6.5**   | 3.15e-17                                    | 5.53e-78                        | Up                        |
| PECAM1    | 3.8       | 1.3       | 1.2       | 1.2       | **2.1**   | 4.69e-07                                    | 7.64e-24                        | Up                        |
| LOC25845  | 2.0       | 1.3       | 1.4       | 1.6       | **2.5**   | 4.47e-06                                    | 1.29e-20                        | Up                        |
| CEND1     | -5.0      | 2.2       | 2.2       | 2.4       | **4.1**   | 4.42e-06                                    | 1.08e-17                        | Up                        |
| GBX2      | -6.3      | 1.0       | 1.5       | 1.3       | **2.8**   | 1.20e-05                                    | 2.59e-13                        | Up                        |
| PGAM2     | -1.7      | 1.9       | 2.4       | 2.5       | **5.0**   | 5.73e-05                                    | 1.72e-11                        | Up                        |
| NR1I2     | 5.6       | -1.5      | -2.3      | -2.9      | **-5.8**  | 8.41e-05                                    | 8.06e-11                        | Down                      |
| GDF5      | -6.1      | 1.3       | 1.3       | 1.2       | **2.9**   | 2.73e-05                                    | 8.22e-11                        | Up                        |
| CXCR2P1   | 2.0       | -2        | -2        | -2.2      | **-4.0**  | 4.52e-04                                    | 1.77e-10                        | Down                      |
| GPR1      | -5.5      | 1.3       | 2.1       | 1.8       | **3.9**   | 1.42e-04                                    | 4.33e-10                        | Up                        |
polarity gene and its downregulation has been implicated in the malignancy of breast [32], prostate [49] and bladder cancers [65]. It has been recently found that lower DLG5 expression is correlated with advanced stages of HCC and essential for invadopodium formation, an event critical to cancer metastasis [26]. It is surprising that our study has identified a stage-III specific upregulation in DLG5. Interestingly, evidence is emerging to lend support to our finding that DLG5 might be tumor-promoting. In a very recent review, Saito et al. [43] reinterpreted published results on cell polarity and cancer, and advanced an alternative perspective on the role of polarity regulators in cancer biology. They argued that both cellular and subcellular polarity would be regulated by DLG5 and related polarity proteins. Subcellular polarity might improve the

Fig. 11 Boxplot of top genes with monotonic expression. These six genes (GABRD, PIGU, NDUFA4L2, CRHBP, FLVCR1, TTC13) showed monotonic trends of expression across the cancer stages, and were top-ranked in both the linear models given by eqns. (1) and (2)

Fig. 12 Boxplot of stage-I specific genes. It is seen that CA9 and WNT7B are both maximally downregulated in stage-I
cellular fitness for proliferation and stemness, thereby causing tumor promotion. Hence cell polarity regulation is anti-tumorigenic and subcellular polarity regulation is pro-tumorigenic, and our analysis has uncovered the protumorigenic upregulated activity of DLG5. ECT2 encodes a guanine nucleotide exchange factor that remains elevated during the G2 and M phase in cellular mitosis. ECT2 is found to be upregulated in lung adenocarcinoma and lung squamous cell carcinoma [66], as well as in invasive breast cancer [52]. NCAPG2 is a component of the condensing II complex and involved in chromosome segregation during mitosis. NCAPG2 level were found to be increased in non-small cell lung cancer, and its overexpression was found to be correlated with lymph node metastasis, thus enabling the use of NCAPG2 as a poor prognostic biomarker in lung adenocarcinoma [61].

GNMT is a methyltransferase that catalyses conversion of S-adenosine methionine to s-adenosyl cysteine. In the absence of GNMT, S-adenosine methionine causes hypermethylation of DNA, which represses GNMT levels and is found in HCC samples [24]. This is an epigenetic mechanism for loss of function of tumor suppressors and our study here confirmed the downregulation of GNMT expression. PRR11 is found to be over-expressed in lungs, and its silencing using siRNA resulted in cell cycle arrest and apoptotic cell death, followed by decreased cell growth and viability [64]. A similar knock out experiment of PRR11 in hilar cholangiocarcinoma cell lines resulted in decreased cellular proliferation, migration, and tumor growth [9]. WDHD1 is a key post-transcriptional regulator of centromeric, and consequently genomic, integrity [23] and its overexpression has been identified as biomarker of acute myeloid leukemia [53], and lung and esophageal carcinomas [44]. C15orf42 has been implicated in nasopharyngeal carcinoma [3]. ORC6L overexpression has been identified as a prognostic biomarker of colorectal cancer possibly by enhancing chromosomal instability [54]. XRCC2 was found to increase locally advanced rectal cancer radioresistance by repairing DNA double-strand breaks and preventing cancer cell apoptosis [40].

Stage-IV specific DEGs (Fig. 15)

GABRD, which was the top gene in the linear models as well, encodes for the delta subunit of the gamma-amino butyric acid receptor. The GABA receptor family was found to be frequently downregulated in cancers, except for GABRD, which was found to be up-regulated. Gross et al. [17] proposed that the GABA receptor gene family might play a role in the proliferation independent differentiation of cancer cells. GBX2 is part of the GBX gene family, which are homeobox containing DNA binding transcription factors. GBX2 is overexpressed in prostate cancer and studies show that expression of GBX2 is required for malignant growth of human prostate cancer [15]. PECAM1 overexpression has been linked to peritoneal recurrence of stage II/III gastric cancer patients [47]. CEND1 has been identified as a cell-cycle protein [50]. PGAM2 is a glycolytic enzyme whose upregulation is essential for tumor cell proliferation [57]. NR1I2 downregulation has been used in constructing a prognostic 9-genes expression signature of gastric cancer [51]. GDF5 has been shown to be a downstream target of the TGF-beta signaling pathway [33], stimulating angiogenesis required for the growth and spread of the cancer. GPR1 has been reported to be involved in promoting cutaneous squamous cell carcinoma migration [12]. Two other stage-IV specific genes, namely the downregulated CXCR2P1, which is a C-X-C motif chemokine receptor 2 pseudogene 1, and LOC25845, are minimally documented in the literature in the context of HCC, other cancers or any other
condition. It is worth mentioning however that CXCR2, a member of the GPCR protein family binding the interleukin IL8, has been reported as an effective non-invasive blood based biomarker for HCC [45]. It is notable that ARHGAP42, a Rho GTPase activating protein, was another key result of Xu et al. [56], finding a place both in their set of 50 hub genes correlated with the BCLC staging of HCC patients, and in the set of 13 hub genes correlated with overall survival of HCC patients. Most of the stage-IV specific genes show contra-regulation (i.e., no clear trend) across cancer stages, and only 15 of the 35 genes revealed a monotonic pattern of expression (highlighted in Table 8). The other 20 genes could be unique to the hallmarks of stage-IV cancer, e.g., processes related to lymph node involvement and/or metastasis.

**Conclusion**

We have developed an original protocol for the stage-wise dissection of the HCC transcriptome. We were able to successfully fit a linear model across cancer stages and detected genes with a strong linear expression trend in the cancer phenotype. These genes were found to effectively separate the control and cancer samples.

**Fig. 14** Boxplot of stage-III specific genes. Except for GNMT, the expression of stage-III specific genes show a peak in stage-III, with the expression trend following an inverted U-shape across the stages. The expression trend is convex and reversed for the downregulated GNMT, with minimum expression in stage-III.
were able to assign 2455 differentially expressed genes into one of four stages and visualized their stage specific expression using boxplots. Using a multi-layered approach, we were able to assess the significance of each stage-specific DEG and narrowed down to a handful of candidate significant stage-specific DEG’s. Our analysis yielded two stage-I specific genes (CA9, WNT7B), two stage-II specific genes (APOBEC3B, FAM186A), ten stage-III specific genes (including DLG5, NCAPG2, GNMT and XRCC2) and 35 stage-IV specific genes (including GABRD and CXCR2P1). Though most of these genes constituted novel findings in the context of HCC, a comprehensive literature search indicated connections with other cancer conditions. The analysis of monotonicity of expression has uncovered two genes with documented HCC connection, namely NDUFA4L2 and CRHBP. Correlation of our analysis with gene signatures based on the BCLC staging system revealed two common genes, namely HSP90AB1 and ARHGAP42. Our study might deepen our understanding of the mechanistic basis of HCC progression, and lay the foundation for the development of HCC diagnosis and treatment strategies. Translational research could transform our results into a panel of biomarkers for early clinical decision-making and

Fig. 15 Boxplot of top 10 stage-IV specific genes. All genes, except NR112 and CXCR2P1, show a smooth increasing expression trend reaching peak expression in stage-IV. In the case of NR112 and CXCR2P1, the trend is reversed, with the expression decreasing smoothly to touch the minimum in stage-IV.
rational drug development. It is straightforward to extend our computational methodology to the stage-based analysis of other cancers to obtain a fuller view of disease initiation, progression, and metastasis.

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Authors’ contributions

AP conceived and designed the study. AS and AP performed the experiments and analyzed the data. AP and AS wrote the manuscript. All authors approved the final manuscript.

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Availability of data and materials

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Ethics approval and consent to participate

All data used in the study is available in the public domain, constituting anonymized patient data provided by the TCGA consortium.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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