NOVEL SIGNIFICANT STAGE-SPECIFIC DIFFERENTIALLY EXPRESSED GENES IN LIVER HEPATOCELLULAR CARCINOMA

Arjun Sarathi¹ and Ashok Palaniappan²*

Depts. of ¹Bioengineering and ²Bioinformatics, School of Chemical and BioTechnology, SASTRA deemed University, Thanjavur, Tamil Nadu 613401. INDIA

*Corresponding author: apalania@scbt.sastra.edu

KEY WORDS: LIHC transcriptomics, HCC stages, stage-specific biomarkers, differentially expressed genes, pairwise contrasts, significance analysis, linear modelling, tumorigenesis, cancer progression, metastasis
ABSTRACT

Liver cancer is among the top deadly cancers worldwide with a very poor prognosis, and the liver is a particularly vulnerable site for metastasis of other cancers. In this study, we developed a novel computational framework for the stage-specific analysis of hepatocellular carcinoma initiation and progression. Using publicly available clinical and RNA-Seq data of cancer samples and controls, we annotated the gene expression matrix with sample stages. We performed a linear modelling analysis of gene expression across all stages and found significant genome-wide changes in gene expression in cancer samples relative to control. Using a contrast against the control, we were able to identify differentially expressed genes (log fold change >2) that were significant at an adjusted p-value < 10E-3. In order to identify genes that were specific to each stage without confounding differential expression in other stages, we developed a full set of pairwise stage contrasts 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. 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 and GNMT, and ten stage-IV specific genes including GABRD, PGAM2 and PECAM1. Of these, only APOBEC3B is an established cancer driver gene. DLG5 was found to be tumor-promoting contrary to the cancer literature on this gene. Further, GABRD, well studied in literature on other cancers, emerged as a stage-IV specific gene. Our findings could be validated using multiple sources of omics data as well as experimentally. The biomarkers identified herein could potentially underpin diagnosis as well as pinpoint drug targets.
INTRODUCTION

Liver cancer is the second most deadly cancer in terms of mortality rate, with a very poor prognosis (Yang et al 2010). 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 (Ferlay et al., 2015). Liver cancer showed the greatest increase in mortality in the last decade for both males (53%) and females (59%) (Cancer Research UK, 2018). Liver hepatocellular carcinoma (LIHC; HCC) is the most common type of liver cancer. 78% of all reported cases of LIHC were due to viral infections (53% Hepatitis B virus and 25% Hepatitis C virus) (Perz et al., 2006). There are several non-viral causes of LIHC as well, mainly aflatoxins and alcohol (Chuang et al., 2009). As shown in Fig. 1, all the factors converge to a common mechanism of genetic alterations that lead to the acquisition of cancer hallmarks (Hanahan and Weinberg, 2011) and the eventual emergence of a cancer cell (Farazi et al., 2006). Genetic alterations constitute the heart of the problem, and studying changes due to these genetic alterations is paramount to understand LIHC. Early 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 LIHC (Xu et al., 2001). The Cancer Genome Atlas (TCGA) research network (2017) have subtyped and identified many potential targets for LIHC 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 (Peng et al., 2015). Zhang et al. (2015) have performed mutation analysis of LIHC, and Yang et al. (2017) 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 LIHC. 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 (Amin et al., 2017). 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 (Palaniappan et al., 2016). A stage-focussed analysis of colorectal cancer transcriptome data yielded negative results vis-a-vis the AJCC staging system (Huo et al., 2017).

METHODS

DATA PREPROCESSING. Normalized and log2-transformed Illumina HiSeq RNA-Seq gene expression data processed by the RSEM pipeline (Li and Dewey, 2011) were obtained from TCGA via the firebrowse.org portal (Broad Institute TCGA Genome Data Analysis Center, 2016). 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 LIHC 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 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 $σ < 1$). Finally, we removed cancer samples from our analysis that were missing stage annotation (value 'NA' in the "pathologic stage"). The data pre-processing was done using R (www.r-project.org).
**LINEAR MODELLING.** 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 (Ritchie et al., 2015). 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 stagewise log fold-change (lfc) coefficients relative to controls. The linear model was subjected to empirical Bayes adjustment to obtain moderated t-statistics (McCarthy and Smyth, 2009). 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 (1990). 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.

**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 |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 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 |lfc| < 2 for all stages. For each remaining gene, we identified the stage that showed the highest |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
(ii)-(iv) P-value of the contrast with respect to other stages < 0.05

To obtain the above p-values (ii) - (iv), we used the contrast matrix shown in Table 2, which was then used as 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 (www.kegg.ac.jp) and Gene Ontology (Ashburner et al., 2000) using kegga 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 (Futreal et al., 2004).

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 LIHC was around 60 years, and the average BMI was about 26, indicating a possible link with ageing and obesity.

The dataset was processed through voom in limma to prepare for linear modelling (Law et al., 2014). At a p-value cutoff of 10E-5, 9618 genes were significant in the linear modelling, implying a strong linear trend in their expression across cancer stages. This was not entirely surprising since one of the hallmarks of cancer phenotype is genome-wide instability (Hanahan and Weinberg, 2011). The linear modelling highlighted top ranked genes, some upregulated in LIHC (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 a progressive net increase in 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 S2. For comparison, a PCA plot of 100 randomly sampled genes (Fig. 7B) failed to show any separation of the cancer and control samples.

The results from the linear modelling were in contrast with those obtained by Huo et al. (2017) and were most likely driven by the inclusion of 51 controls in our study. These positive results provided the impetus to pursue stage-driven analysis. Given the conventional AJCC staging, gene expression differences would play a major role in driving the cancer progression. To identify the stage-specific differentially expressed genes, we applied the first contrast matrix (Table 2) and constructed the four-bit stage string of each gene. Based on the stage strings, we binned all the genes, and the string-specific gene lists corresponding to all the partitions in the Venn diagram (Fig. 4) is made available 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 6. 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 7). Fig. 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 ten topranked by significance in the linear modelling.

A heatmap of the lfc expression of stage-specific genes across different stages was visualized (Fig. 10A) and revealed systematic variation in expression relative to control on a gradient from blue (downregulated) to red (overexpressed). The map was clustered on the basis of differential expression (i.e, lfc) both across stages and across features (i.e, genes) (Fig. 10B). 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.

**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.

To identify the biological processes specific to each stage, we used the genes with maximal llogFCI 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 8) 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-I 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.

The top ten linear model genes (Table 5) and all the stage-specific differentially expressed genes (Table 10) were analyzed with respect to the existing literature. Three C-type lectin domain proteins (CLEC4M, CLEC1B, CLEC4G) were detected in the top ten genes of linear modelling across
stages. 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 (Ho et al., 2015). On screening the top 200 linear model genes against cancer
driver genes in the Cancer top 200 Gene Census, only four genes were found, namely BUB1B,
CDKN2A, EZH2, and RECQL4.

Stage-I specific DEGs (Fig. 11). 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 (Li et al., 2017). 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 (Arensman et al., 2014). Surprisingly, both CA9 and
WNT7B are downregulated in LIHC, most so in stage-I, contrary to their role in other cancers. A
concrete interpretation of the role of these genes in LIHC awaits appropriately designed
experimental studies.

It is pertinent to ask the following question here: which genes are essential for the initiation of
LIHC? Clearly these genes would be differentially expressed in stage I relative to control. All
significantly differentially expressed genes with maximal lfc in stage-I would be the best
candidates for genes involved in the initiation of LIHC. These 122 genes are provided in the
Supplementary File S3.

Stage-II specific DEGs (Fig. 12). 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 was found to be highly mutated in Bladder, lung, cervix, neck, and head cancers as well
(Burns et al., 2013). Here APOBEC3B is upregulated possibly conferring a gain-of-function
comparable to that achieved by a mutation mechanism. 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 (Timofeeva et al., 2015).

Stage-III specific DEGs (Fig. 13). C12orf48, also known as PARI, participates in the homologous
recombination pathway of DNA repair, and its overexpression has been reported in pancreatic
cancer(O'Connor et al., 2013). Further PARI was recently identified as a transcriptional target of
FOXM1 (Zhang et al., 2018), which is a well-validated upregulated gene in LIHC (Ho et al., 2015).
DLG5 is a cell polarity gene and its downregulation has been implicated in the malignancy of
breast (Liu et al., 2017), prostate (Tomiyama et al., 2015) and bladder cancers (Zhou et al., 2015). 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 (Ke et al., 2017). 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. (2018) 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 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 pro-tumorigenic
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 (Zhou et al., 2017), as well as in invasive breast
cancer (Wang et al., 2017). 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 over-expression was found to be correlated with lymph node metastasis, thus enabling the use of NCAPG2 as a poor prognostic biomarker in lung adenocarcinoma (Zhan et al., 2017). 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 (Huidobro et al., 2013). 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 (Zhao 2015). A similar knock out experiment of PRR11 in hilar cholangiocarcinoma cell lines resulted in decreased cellular proliferation, migration, and tumor growth (Chen et al., 2015). WDHD1 is a key post-transcriptional regulator of centromeric, and consequently genomic, integrity (Hsieh et al., 2011) and its overexpression has been identified as biomarker of acute myeloid leukemia (Wermke et al., 2015), and lung and esophageal carcinomas (Sato et al., 2010). C15orf42 has been implicated in nasopharyngeal carcinoma (An et al., 2015). ORC6L overexpression has been identified as a prognostic biomarker of colorectal cancer possibly by enhancing chromosomal instability (Xi et al., 2008). XRCC2 was found to increase locally advanced rectal cancer radioresistance by repairing DNA double-strand breaks and preventing cancer cell apoptosis (Qin et al 2015).

Stage-IV specific DEGs (Fig. 14). GABRD, which is the top gene in the linear model as well, encodes for the delta subunit of the gamma-aminobutyric 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. (2015) 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 (Gao et al., 1998). PECAM1 overexpression has been linked to peritoneal recurrence of stage II/III gastric cancer patients (Terashima et al., 2017). CEND1 has been identified as a cell-cycle protein (Tsioras et al., 2013). PGAM2 is a glycolytic enzyme whose upregulation is essential for tumor cell proliferation (Xu et al., 2014). NR1I2 downregulation has been used in constructing a prognostic 9-genes expression signature of gastric cancer (Wang et al., 2017). GDF5 has been shown to be a downstream target of the TGF-beta signaling pathway (Margheri et al., 2012), 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 (Farsam et al., 2016). Two more stage-IV specific genes, namely CXCR2P1, which is a C-X-C motif chemokine receptor 2 pseudogene 1, and LOC25845, are undocumented in the literature in the context of LIHC, other cancers or any other condition.

CONCLUSION

We have developed an original protocol for the stagewise dissection of the LIHC 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. We 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 and ten stage-IV specific genes. Though all these genes except APOBEC3B are novel, a literature search indicated that most of the genes have a cancer connection (albeit not with LIHC). Experimental validation would be useful to translate these results into a panel of biomarkers for clinical use and 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.

ACKNOWLEDGMENTS:

We would like to thank SASTRA deemed University, for infrastructure and computing support.

SUPPLEMENTARY INFORMATION:

All data and results are provided in the supplementary information (10.6084/m9.figshare.6455024).

REFERENCES

1. Amin, M.B., Greene, F.L., Edge, S.B., Compton, C.C., Gershenwald, J.E., Brookland, R.K., Meyer, L., Gress, D.M., Byrd, D.R. and Winchester, D.P., (2017). The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more "personalized" approach to cancer staging. CA: a cancer journal for clinicians, 67(2), pp.93-99.

2. An F, Zhang Z, Xia M (2015). Functional analysis of the nasopharyngeal carcinoma primary tumor-associated gene interaction network. Mol Med Rep 12(4):4975-80. doi: 10.3892/mmr.2015.4090

3. Arensman, M.D., Kovochich, A.N., Kulikauskas, R.M., Lay, A.R., Yang, P.T., Li, X., Donahue, T., Major, M.B., Moon, R.T., Chien, A.J. and Dawson, D.W., (2014). WNT7B mediates autocrine Wnt/β-catenin signaling and anchorage-independent growth in pancreatic adenocarcinoma. Oncogene, 33(7), p.899.

4. Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T. and Harris, M.A., (2000). Gene Ontology: tool for the unification of biology. Nature genetics, 25(1), p.25.

5. Broad Institute TCGA Genome Data Analysis Center (2016): Analysis-ready standardized TCGA data from Broad GDAC Firehose 2016_01_28 run. Broad Institute of MIT and Harvard. Dataset. https://doi.org/10.7908/C11G0KM9

6. Burns, M. B., Temiz, N. A., and Harris, R. S. (2013). Evidence for APOBEC3B mutagenesis in multiple human cancers. Nature genetics, 45(9), 977.

7. Cancer Research UK, http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer, Accessed 01/2018

8. Chen, Y., Cha, Z., Fang, W., Qian, B., Yu, W., Li, W., Yu, G. and Gao, Y., (2015). The prognostic potential and oncogenic effects of PRR11 expression in hilarcholangiocarcinoma. Oncotarget, 6(24), p.20419.

9. Chuang, S. C., La Vecchia, C., and Boffetta, P. (2009). Liver cancer: descriptive epidemiology and risk factors other than HBV and HCV infection. Cancer letters, 286(1), 9-14.

10. Farazi, P. A., and DePinho, R. A. (2006). Hepatocellular carcinoma pathogenesis: from
11. Farsam, V., et al. (2016). Senescent fibroblast-derived Chemerin promotes squamous cell carcinoma migration. Oncotarget 7(50):83554-83569. doi: 10.18632/oncotarget.13446.

12. Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebulo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer 136, E359–E386.

13. Futreal, P.A., Coin, L., Marshall, M., Down, T., Hubbard, T. et al. (2004) A census of human cancer genes. Nat Rev Cancer 4: 177–183.

14. Gao, A. C., Lou, W., and Isaacs, J. T. (1998). Down-regulation of homeobox gene GBX2 expression inhibits human prostate cancer clonogenic ability and tumorigenicity. Cancer research, 58(7), 1391-1394.

15. Gross, A. M., Kreisberg, J. F., and Ideker, T. (2015). Analysis of matched tumor and normal profiles reveals common transcriptional and epigenetic signals shared across cancer types. PloS one, 10(11), e0142618.

16. Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell 144(5), 646-674.

17. Ho, D. W. H., Kai, A. K. L., and Ng, I. O. L. (2015). TCGA whole-transcriptome sequencing data reveals significantly dysregulated genes and signaling pathways in hepatocellular carcinoma. Frontiers of medicine, 9(3), 322-330.

18. Hochberg Y., and Benjamini Y. (1990) More powerful procedures for multiple significance testing. Stat Med 9: 811–818.

19. Hsieh CL, et al. (2011). WDHD1 modulates the post-transcriptional step of the centromeric silencing pathway. Nucleic Acids Res 39(10):4048-62. doi: 10.1093/nar/gkq1338.

20. Huidobro, C., Toraño, E.G., Fernández, A.F., Urduinguio, R.G., Rodríguez, R.M., Ferrero, C., Martínez-Camblor, P., Boix, L., Bruix, J., García-Rodríguez, J.L. and Varela-Rey, M., (2013). A DNA methylation signature associated with the epigenetic repression of glycine N-methyltransferase in human hepatocellular carcinoma. Journal of Molecular Medicine, 91(8), pp.939-950.

21. Huo T, Canepa R, Sura A, Modave F, and Gong Y (2017) Colorectal cancer stages transcriptome analysis. PLoS ONE 12(11): e0188697.

22. Ke Y., et al. (2017). Discs large homolog 5 decreases formation and function of invadopodia in human hepatocellular carcinoma via Girdin and Tks5. Int J Cancer. 141(2):364-376. doi: 10.1002/ijc.30730.

23. Killian A, et al. (2004). Inactivation of the RRB1-Pescadillo pathway involved in ribosome biogenesis induces chromosomal instability. Oncogene 23(53):8597-602.

24. Law, C. W., Chen, Y., Shi, W., and Smyth, G. K. (2014). voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome biology, 15(2), R29.
25. Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics, 12(1), 323.

26. Li, G., Feng, G., Zhao, A., Péoc’h, M., Cottier, M., and Mottet, N. (2017). CA9 as a biomarker in preoperative biopsy of small solid renal masses for diagnosis of clear cell renal cell carcinoma. Biomarkers, 22(2), 123-126

27. Liu J., et al. (2017). Loss of DLG5 promotes breast cancer malignancy by inhibiting the Hippo signaling pathway. Sci Rep. 7:42125. doi: 10.1038/srep42125.

28. Margheri F, et al. (2012). GDF5 regulates TGFß-dependent angiogenesis in breast carcinoma MCF-7 cells: in vitro and in vivo control by anti-TGFß peptides. PLoS One 7(11):e50342. doi: 10.1371/journal.pone.0050342

29. McCarthy, D. J., and Smyth, G. K. (2009). Testing significance relative to a fold-change threshold is a TREAT. Bioinformatics 25, 765-771.

30. O'Connor KW, et al. (2013). PARI overexpression promotes genomic instability and pancreatic tumorigenesis. Cancer Res 73(8):2529-39. doi: 10.1158/0008-5472.CAN-12-3313.

31. Palaniappan, A., Ramar, K., and Ramalingam, S. (2016). Computational identification of novel stage-specific biomarkers in colorectal cancer progression. PloS one, 11(5), e0156665.

32. Peng, L., Bian, X.W., Li, D.K., Xu, C., Wang, G.M., Xia, Q.Y. and Xiong, Q. (2015) Large-scale RNA-Seq Transcriptome Analysis of 4043 Cancers and 548 Normal Tissue Controls across 12 TCGA Cancer Types. Scientific Reports 5:13413 | DOI: 10.1038/srep13413

33. Perz, J. F., Armstrong, G. L., Farrington, L. A., Hutin, Y. J., and Bell, B. P. (2006). The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. Journal of hepatology, 45(4), 529-538.

34. Qin, C. J., Song, X. M., Chen, Z. H., Ren, X. Q., Xu, K. W., Jing, H., and He, Y. L. (2015). XRCC2 as a predictive biomarker for radioresistance in locally advanced rectal cancer patients undergoing preoperative radiotherapy. Oncotarget, 6(31), 32193.

35. Ritchie, M.E., Phipson, B., Wu,D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015) limma powers differential expression analyses forRNA-sequencing and microarray studies Nucleic Acids Research, 43(7) doi: 10.1093/nar/gkv007

36. Saito Y, Desai RR, Muthuswamy SK. (2018). Reinterpreting polarity and cancer: The changing landscape from tumor suppression to tumor promotion. Biochim Biophys Acta 1869(2):103-116. doi: 10.1016/j.bbabio.2017.12.001.

37. Sato N, et al. (2010) Activation of WD repeat and high-mobility group box DNA binding protein 1 in pulmonary and esophageal carcinogenesis. Clin Cancer Res 16(1):226-39. doi: 10.1158/1078-0432.CCR-09-1405

38. Siegel, R. L., Miller, K. D., and Jemal, A. (2018). Cancer statistics, 2018. CA: a cancer journal for clinicians, 68(1), 7-30.

39. TCGA Research Network (2017). Comprehensive and integrative genomic characterization
of hepatocellular carcinoma. Cell, 169(7), pp.1327-1341.

40. Terashima M, et al. (2017). TOP2A, GGH, and PECAM1 are associated with hematogenous, lymph node, and peritoneal recurrence in stage II/III gastric cancer patients enrolled in the ACTS-GC study. Oncotarget 8(34):57574-57582. doi: 10.18632/oncotarget.15895

41. Timofeeva M.N., et al. (2015). Recurrent Coding Sequence Variation Explains Only A Small Fraction of the Genetic Architecture of Colorectal Cancer. Sci Rep. 5:16286. doi: 10.1038/srep16286.

42. Tomiyama L, Sezaki T, Matsu M, Ueda K, Kioka N. (2015). Loss of Dlg5 expression promotes the migration and invasion of prostate cancer cells via Girdin phosphorylation. Oncogene 34(9):1141-9. doi: 10.1038/onc.2014.31.

43. Tsioras K, Papastefanaki F, Politis PK, Matsas R, Gaitanou M (2013). Functional Interactions between BM88/Cend1, Ran-binding protein M and Dyrk1B kinase affect cyclin D1 levels and cell cycle progression/exit in mouse neuroblastoma cells. PLoS One 8(11):e82172. doi: 10.1371/journal.pone.0082172

44. Wang Z, Chen G, Wang Q, Lu W, Xu M. Identification and validation of a prognostic 9-genes expression signature for gastric cancer. Oncotarget 8(43):73826-73836. doi: 10.18632/oncotarget.17764

45. Wang, H. K., Liang, J. F., Zheng, H. X., and Xiao, H. (2017). Expression and prognostic significance of ECT2 in invasive breast cancer. Journal of clinical pathology, jclinpath-2017.

46. Wermke M, et al. (2015). RNAi profiling of primary human AML cells identifies ROCK1 as a therapeutic target and nominates fasudil as an antileukemic drug. Blood 125(24):3760-8. doi: 10.1182/blood-2014-07-590646

47. Xu Y, et al. (2014). Oxidative stress activates SIRT2 to deacetylate and stimulate phosphoglycerate mutase. Cancer Res 74(13):3630-42. doi: 10.1158/0008-5472.CAN-13-3615

48. Xu, X.R., Huang, J., Xu, Z.G, Qian, B.Z., Zhu, Z.D., Yan, Q., Cai, T., Zhang, X., Xiao, H.S., Qu, J. and Liu, F., (2001). Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. Proceedings of the National Academy of Sciences, 98(26), pp.15089-15094.

49. Yang, H., Zhang, X., Cai, X.Y., Wen, D.Y., Ye, Z.H., Liang, L., Zhang, L., Wang, H.L., Chen, G and Feng, Z.B., (2017). From big data to diagnosis and prognosis: gene expression signatures in liver hepatocellular carcinoma. PeerJ, 5, p.e3089

50. Yang, J. D. and Roberts, L. R. (2010) Hepatocellular carcinoma: a global view, Nat. Rev. Gastroenterol. Hepatol. 7, 448–458 .doi:10.1038/nrgastro.2010.100

51. Zhan, P., Xi, G. M., Zhang, B., Wu, Y., Liu, H. B., Liu, Y. F., Xu, W.J., Zhu, Q., Cai, F., Zhou, Z.J. and Miu, Y. Y. (2017). NCAPG2 promotes tumour proliferation by regulating G2/M phase and associates with poor prognosis in lung adenocarcinoma. Journal of cellular and molecular medicine, 21(4), 665-676.
52. Zhang Y, et al. (2018). PARI functions as a new transcriptional target of FOXM1 involved in gastric cancer development. Int J Biol Sci 14(5):531-541. doi: 10.7150/ijbs.23945

53. Zhang, Y., Qiu, Z., Wei, L., Tang, R., Lian, B., Zhao, Y., He, X. and Xie, L., (2014). Integrated analysis of mutation data from various sources identifies key genes and signaling pathways in hepatocellular carcinoma. PloS one, 9(7), p.e100854.

54. Zhao, Q. (2015). RNAi-mediated silencing of praline-rich gene causes growth reduction in human lung cancer cells. International journal of clinical and experimental pathology, 8(2), 1760

55. Zheng, D., Decker, K.F., Zhou, T., Chen, J., Qi, Z., Jacobs, K., Weilbaecher, K.N., Corey, E., Long, F. and Jia, L., (2013). Role of WNT7B-induced noncanonical pathway in advanced prostate cancer. Molecular Cancer Research, 11(5), pp.482-493.

56. Zhou Z, et al. (2015). Methylation-mediated silencing of Dlg5 facilitates bladder cancer metastasis. Exp Cell Res. 331(2):399-407. doi: 10.1016/j.yexcr.2014.11.015

57. Zhou, S., Wang, P., Su, X., Chen, J., Chen, H., Yang, H., Fang, A., Xie, L., Yao, Y. and Yang, J., (2017). High ECT2 expression is an independent prognostic factor for poor overall survival and recurrence-free survival in non-small cell lung adenocarcinoma. PloS one, 12(10), p.e0187356.
Figure 1 Major causative pathways of hepatocarcinogenesis. All pathways converge to progressive genomic alterations, leading a normal cell to acquire the hallmarks of cancer.
Figure 2 TCGA 'Hybridization REF' Barcode. The first 10 characters constitute an anonymized unique patient identifier and the following two characters denote whether the sample is tumor or normal.
Figure 3 Design matrices. A, In the linear modeling, the control samples served as the baseline expression (intercept) of each gene against which the stage-specific expression was estimated. B, the design matrix for the contrasts analysis.
Figure 4. A Venn representation of the pairwise stages contrasts. A gene could be differentially expressed in any combination of the four stages and this could be represented by a 4-bit string, one bit for each stage. For e.g, '1111' at the overlap of all four stages would be assigned to genes that are differentially expressed in all four stages.
Figure 5 **Boxplots of top 9 linear model genes.** For each gene, notice that the trend in expression could be overexpression or downregulations relative to the control. For e.g, GABRD, PLVAP, CXorf36, CDH13 and UBE2T are overexpressed, while CLEC4M, CLEC1B, BMP10, and CLEC4G are downregulated. It could be seen that a linear trend does not imply maximal |lfc| in stage 4, as illustrated most clearly in the case of UBE2T.
Figure 6 **Boxplots illustrating stage-specificity of differentially expressed genes.** Extremal expression in a stage could be either maximal expression or minimal expression relative to the control and all other stages, and could be termed maximal differential expression. Here we show genes with maximal differential expression in stage-I (WDR72; minimum expression), stage-II (GLI4, maximum expression; COLEC11, minimum expression), stage-III (CKAP2; maximum expression), and stage-IV (MAPK11; maximum expression).
Figure 7. **Principal components analysis of cancer vs control.** A, The first two principal components of the top 100 genes from linear modeling are plotted. It could be seen that control samples (red) clustered independent of the cancer samples (colored by stage). B, The same analysis repeated with 100 random genes failed to effect a clustering of the control samples relative to the cancer samples.
Figure 8 Venn illustration of the size of each 4-bit string. The number of genes having each pattern of differential expression are shown.
Figure 9 Volcano plot of final 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 away from the origin, indicating significance and effect size.
Figure 10 **Heatmaps of final stage-specific 24 genes.** A, heatmap generated from the lfc values of all the stage-specific genes (arranged stagewise). Log fold changes up to sixfold are seen, indicating 64 times differential expression with respect to control. B, Clustered representation of the stagewise gene expression based on differential expression (corresponding to lfc).
Figure 11 **Boxplot of stage-I specific genes.** It is seen that CA9 and WNT7B are both maximally downregulated in stage-I.

Figure 12 **Boxplot of stage-II specific genes.** It is seen that both APOBEC3B and FAM186A expression are maximum in stage-II, following an inverted U-shape.
Figure 13 **Boxplot of stage-III specific genes.** Except for GNMT, the expression of all other genes show a ** U-shape. The expression trend is reversed for the downregulated GNMT, following a U-shape.
Figure 14 **Boxplot of top 10 stage 4 specific genes.** All genes, except NR1I2 and CXCR2P1, show a smooth increasing trend of expression reaching its maximum in stage-IV. For NR1I2 and CXC2RP1, the trend is reversed, with a smooth decreasing expression reaching its minimum in stage-IV.
### Table 1. Contrast matrix with control. Each stage (indicated by '1') is contrasted against the control (indicated by '-1') in turn.
| Control | Stage2-Stage1 | Stage3-Stage1 | Stage3-Stage2 | Stage4-Stage1 | Stage4-Stage2 | Stage4-Stage3 |
|---------|---------------|---------------|---------------|---------------|---------------|---------------|
| 0       | 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             |

**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.
| 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     |
| 3          | -                  | 3     |
| 4A         | T(any) N1 M0       | 1     |
| 4B         | T(any) N(any) M1   | 2     |
| 4          | -                  | 2     |
| CONTROL    | -                  | 50    |
| NA         | -                  | 24    |

Table 3 AJCC Cancer staging. The correspondence between the AJCC staging and the TCGA staging for LIHC is noted, along with the number of LIHC cases in each stage in the TCGA dataset. Control indicates the number of normal tissue control samples, and NA denotes cases where the stage information is unavailable.
| 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 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.
| 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      |

**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.
| 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    |

**Table 6 Number of genes in each step of the significance analysis.** Differential expression is defined with respect to a threshold |logFC| = 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').
| 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            | NR112        |
|              |                  | NCAPG2           | GDF5         |
|              |                  | GNMT             | CXCR2P1      |
|              |                  | PRR11            | GPR1         |
|              |                  |                  | MUSTN1       |
|              |                  |                  | EHD2         |
|              |                  |                  | LOC143188    |
|              |                  |                  | HIST3H2BB    |
|              |                  |                  | CA12         |
|              |                  |                  | CDX1         |
|              |                  |                  | MYO16        |
|              |                  |                  | CPE          |
|              |                  |                  | LPPR3        |
|              |                  |                  | ZMYND12      |
|              |                  |                  | KCNF1        |
|              |                  |                  | GPR126       |
|              |                  |                  | MCCD1        |
|              |                  |                  | GABRB2       |
|              |                  |                  | SNCB         |
|              |                  |                  | TRIM50       |
|              |                  |                  | MT3          |
|              |                  |                  | KCNQ2        |
|              |                  |                  | DUXA         |
|              |                  |                  | C14orf72     |
|              |                  |                  | ECEL         |
|              |                  |                  | FOXE         |
|              |                  |                  | MYH13        |
|              |                  |                  | ARHGAP42     |
|              |                  |                  | BMP7         |

**Table 7 Final set of highlighted genes in each stage.** The genes in each stage are ordered by increasing adjusted p-values of the linear modelling analysis.
### Table 8 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     |
| Stage 4 | Bile secretion                                                                    | 2.479e-06     |
|        | ABC transporters                                                                  | 7.146e-06     |
|        | Primary bile acid biosynthesis                                                    | 2.357e-03     |
|        | AGE-RAGE signalling pathway in diabetic complications                              | 3.024e-02     |
| 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) |
|-------|-----------|-----------|-----------|-----------|-----------|-----------------------------------------------|--------------------------------|---------------------------|
| 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                      |
| 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                        |
| 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                        |
| Cl2orf48 | -1.9   | 2.8       | 3.2       | **3.7**   | 2.4       | 5.88e-43                                      | 1.19e-43                       | Up                        |
| Cl5orf42 | -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                        |
| 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                        |

**Table 9 Stage specific genes and parameters.** The log-expressions ($\beta$'s) of each gene from our analysis are shown, along with adjusted p-values with respect to control and the linear model, and the inferred regulation status of the gene in LIHC. The stage-specificity of the genes are emphasized.