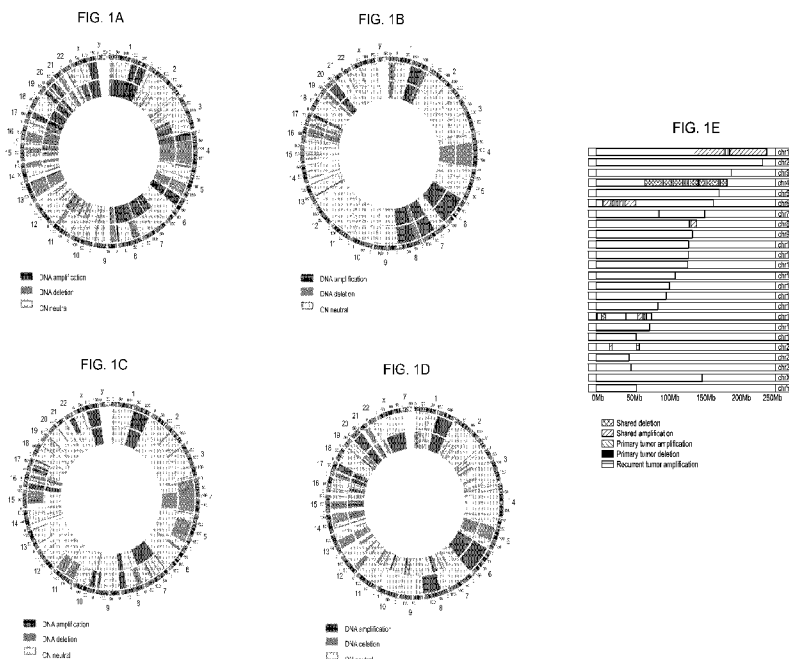




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[Continued on next page]

(54) **Title:** METHODS FOR TREATING HEPATOCELLULAR CARCINOMA

(57) **Abstract:** This application provides methods of treating hepatocellular carcinoma in a patient, methods of evaluating the recurrence risk profile of a hepatocellular carcinoma, and diagnostic agents for the diagnosis of hepatocellular carcinoma.



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METHODS OF TREATING HEPATOCELLULAR CARCINOMA**DESCRIPTION****Background**

[001] Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and third leading cause of cancer deaths worldwide, with hepatitis B virus a major etiological factor. *El-Serag, H.B. & Rudolph, K.L.*, Gastroenterology 132:2557-2576 (2007); *Parkin, D.M.*, Int J Cancer 118, 3030-3044 (2010). Aside from sorafenib which is only marginally effective in a small HCC patient population, there is no approved treatment for HCC. Patients have limited treatment options. Surgical resection, ablation, or orthotopic liver transplantation (OLT) are viable for certain patients, though recurrence rates can be as high as 40% following these procedures. *Chang, M.H. et al.*, J. Natl. Cancer Inst. 101:1348-1355 (2009); *Marsh, J.W. et al.*, Hepatology 26:444-450 (1997). HCC recurrence rates after OLT are 20-40% in patients who meet clinical criteria such as the Milan, Toronto, or UCSF criteria. *Chang et al.*, J Natl Cancer Inst 101:1348-1355 (2009); *Marsh et al.*, Hepatology 26:444-450 (1997). Cases of donor transmitted malignancies in cadaveric organ transplants are very rare and arise either *de novo* in the transplanted organ or are preexisting in the donor organ. In both cases tumor origin is independent of the recipient's primary cancer. The most prevalent occurrences of recurrent HCC cases post-OLT are tumors derived from the recipient, with recurrent tumor genetic composition matching the primary tumor. Thus, there is a need for determining the risk for recurrence based on the properties of the primary tumor.

[002] Studies have shown varied levels of consistency for classifying patients with high risk for HCC recurrence using clinicopathologic variables such as tumor size, vascular invasion, tumor state, tumor grade, and alpha-fetoprotein levels, among others. *Clavien et al.*, Lancet Oncol. 13(1):e11-e22 (2012). In a deadly disease with no established adjuvant treatments, substantial shortage of donor organs, and high financial burden, identifying

eligible patients for OLT with low recurrence risk at the molecular level accompanied by current clinical criteria, has great potential to significantly improve patient clinical outcomes.

SUMMARY

[003] In accordance with the description, a method of treating hepatocellular carcinoma in a patient comprises: (a) obtaining a hepatocellular carcinoma sample from the patient; (b) determining whether the sample has a normal level of HERC5 mRNA expression; and (c) qualifying the patient for an orthotopic liver transplant if the sample has a normal level of HERC5 mRNA expression. In one embodiment, the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a historical control group. In some instances, the historical control comprises data from at least 10, 25, or 50 individuals.

[004] In one embodiment, the historical control group has normal subjects. In another embodiment, the historical control group has patients who had a hepatocellular carcinoma that did not recur within two years. In one embodiment, the HERC5 mRNA level in a patient sample level is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical control group. In another mode, the HERC5 mRNA level in a patient sample is designated as normal if it is equal to or higher than one standard deviation below the mean of the historical control group.

[005] In a further embodiment, the historical control group has patients who had a hepatocellular carcinoma that recurred within two years. In one aspect, the HERC5 mRNA level in a patient sample is designated as normal if it is equal to or higher than one standard deviation above the mean of the historical control group.

[006] In another aspect, the historical control group has patients who have had hepatocellular carcinoma. In one embodiment, the HERC5 mRNA level in a patient sample is designated as normal if it is higher than the median of the historical group.

[007] According to one method, the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a sample of normal tissue obtained from the patient. In one embodiment, the normal tissue is liver tissue. In one mode, the level of HERC5 mRNA in the hepatocellular carcinoma sample is higher than 80% of the level in the normal tissue sample and the patient qualifies for an orthotopic liver transplant. In another mode, the level of HERC5 mRNA in the hepatocellular carcinoma sample is lower than 50% of the level in the normal tissue sample and the patient is disqualified from an orthotopic liver transplant.

[008] In one embodiment, the patient does not qualify for sorafenib. In another embodiment, the patient was treated with sorafenib and the treatment was unsuccessful.

[009] In one embodiment, the hepatocellular carcinoma sample has a HERC5 DNA deletion and the patient is not qualified for an orthotopic liver transplant. In another embodiment, the hepatocellular carcinoma sample has HERC5 mRNA underexpression and the patient is not qualified for an orthotopic liver transplant.

[010] In a further embodiment, the decision to qualify a patient for an orthotopic liver transplant also includes an evaluation of clinical criteria. In one aspect, the clinical criteria comprise at least one or more of the following: size of the lesion, the number of lesions, whether there are extrahepatic manifestations, whether there is vascular invasion, whether the HCC was poorly differentiated on biopsy, tumor state, tumor grade, and alpha-fetoprotein levels. In another aspect, the clinical criteria comprise (a) a single lesion not exceeding 5 cm or no more than 3 lesions, none greater than 3 cm; and (b) no macrovascular invasion. In a further aspect, the clinical criteria comprise UCSF criteria and include: (a) a single lesion not greater than 6.5 cm; or (b) no more than 3 lesions, none of which exceed 4.5 cm and whose total tumor diameter does not exceed 8 cm.

[011] In an additional embodiment, the clinical criteria comprise the Toronto criteria and include any tumor size or number and no systemic symptoms as a result of HCC and histological-based exclusion of poorly differentiated HCC (beyond Milan tumors only). In a further mode, the clinical criteria comprise the Hangzhou criteria and include: a) total tumor diameter less than or equal to 8 cm; (b) total tumor diameter more than 8 cm, with histopathologic grade I or II and preoperative AFP level less than or equal to 400 ng/ml.

[012] In one embodiment, the α -fetoprotein concentration is less than 400 ng/mL (with a lower level indicating a potential positive outcome after OLT).

[013] In one mode, a patient with a normal level of HERC5 mRNA is given priority on a transplant waiting list over any hepatocellular carcinoma patient without a normal level of HERC5 mRNA. In one embodiment, the patient receives a liver transplant.

[014] In accordance with the description, a method of evaluating the recurrence risk profile of a hepatocellular carcinoma comprises: (a) providing a hepatocellular carcinoma sample; (b) determining *in vitro* whether the sample has a normal level of HERC5 mRNA expression; (c) qualifying the hepatocellular carcinoma as having a low risk of recurrence if the sample has a normal level of HERC5 mRNA expression and the determination is made in conjunction with at least one clinical criteria. In one embodiment, the hepatocellular carcinoma has a low risk of recurrence after an orthotopic liver transplant. In some instances, the historical control comprises data from at least 10, 25, or 50 individuals.

[015] In one embodiment, the historical control group has normal subjects. In another embodiment, the historical control group has patients who had a hepatocellular carcinoma that did not recur within two years. In one embodiment, the HERC5 mRNA level in a patient sample is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical control group. In another mode, the HERC5

mRNA level in a patient sample is designated as normal if it is equal to or higher than one standard deviation below the mean of the historical control group.

[016] In a further embodiment, the historical control group has patients who had a hepatocellular carcinoma that recurred within two years. In one aspect, the HERC5 mRNA level in a patient sample is designated as normal if it is equal to or higher than one standard deviation above the mean of the historical control group.

[017] In another aspect, the historical control group has patients who have had hepatocellular carcinoma. In one embodiment, the HERC5 mRNA level in a patient sample is designated as normal if it is higher than the median of the historical group.

[018] According to one method, the level of HERC5 mRNA level in the sample is compared to the level of mRNA of HERC5 in a sample of normal tissue obtained from the patient. In one embodiment, the normal tissue is liver tissue. In one mode, the level of mRNA of HERC5 in the hepatocellular carcinoma sample is higher than 80% of the level in the normal tissue sample and the patient qualifies for an orthotopic liver transplant. In another mode, the level of mRNA of HERC5 in the hepatocellular carcinoma sample is lower than 50% of the level in the normal tissue sample and the patient is disqualified from an orthotopic liver transplant.

[019] In one embodiment, the patient does not qualify for sorafenib. In another embodiment, the patient was treated with sorafenib and the treatment was unsuccessful.

[020] In one embodiment, the hepatocellular carcinoma sample has HERC5 DNA deletion and the patient is not qualified for an orthotopic liver transplant. In another embodiment, the hepatocellular carcinoma sample has mRNA HERC5 underexpression and the patient is not qualified for an orthotopic liver transplant.

[021] In a further embodiment, the decision to qualify a patient for an orthotopic liver transplant also includes an evaluation of clinical criteria. In one aspect, the clinical

criteria comprise at least one of the size of the lesion, the number of lesions, whether there are extrahepatic manifestations, whether there is vascular invasion, whether the HCC was poorly differentiated on biopsy, tumor state, tumor grade, and alpha-fetoprotein levels. In another aspect, the clinical criteria comprise (a) a single lesion not exceeding 5 cm or no more than 3 lesions, none greater than 3 cm; and (b) no macrovascular invasion. In a further aspect, the clinical criteria comprise UCSF criteria and include: (a) a single lesion not greater than 6.5 cm; or (b) no more than 3 lesions, none of which exceed 4.5 cm and whose total tumor diameter does not exceed 8 cm.

[022] In an additional embodiment, the clinical criteria comprise the Toronto criteria and include any tumor size or number and no systemic symptoms as a result of HCC and histological-based exclusion of poorly differentiated HCC (beyond Milan tumors only) In a further mode, the clinical criteria comprise the Hangzhou Zhou criteria and include a) total tumor diameter less than or equal to 8 cm; (b) total tumor diameter more than 8 cm, with histopathologic grade I or II and preoperative AFP level less than or equal to 400 ng/mL.

[023] In one embodiment, the α -fetoprotein concentration is less than 400 ng/mL (with a lower level indicating a potential positive outcome after OLT).

[024] In one mode, a patient with a normal level of HERC5 mRNA is given priority on a transplant waiting list over any hepatocellular carcinoma patient without a normal level of HERC5 mRNA. In one embodiment, the patient receives a liver transplant.

[025] Another embodiment includes the use of a HERC5 forward and reverse primer, each of which is generated from the mRNA of HERC5, for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.

[026] A further embodiment includes the use of a HERC5-specific probe for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.

[027] An additional embodiment includes the use of a HERC5 nucleic acid sequence as a quantitative control for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.

[028] Additional objects and advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice. The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[029] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

[030] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) and together with the description, serve to explain the principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[031] Figures 1A-D shows somatic CN amplifications (red) or deletions (green) identified in the primary (inner track) and recurrent (outer track) tumors of **A)** HCC1, **B)** HCC4, **C)** HCC5, and **D)** HCC11; **E)** both shared and unique regions of CN amplifications or deletions in 3/4 patients across the genome. Color code is as follows: unique to primary tumors (red=amplification; blue=deletion); unique to recurrent tumors

(brown=amplification); and shared by the primary and recurrent tumors (green=amplification; purple=deletion).

[032] Figure 2 shows Wnt/ β -catenin signaling and directly related pathways affected by genetic-driven and/or gene expression activation or suppression in primary and/or recurrent tumors of 4 HCC patients.

[033] Figures 3A-F show that *HERC5* mRNA expression level predicts tumor recurrence, overall survival, and progression-free survival in HCC using two independent studies. **A)** Gene expression $\log_2$ fold change distribution between liver tissue from normal healthy controls (red; n=239) and tumor biopsies from HCC patients (green; n=247), where the blue line indicates mean of normals - 2SD; **B)** Kaplan-Meier (KM) curves comparing the low *HERC5* mRNA expression group (n=62) to the high *HERC5* mRNA expression group (n=180) with HCC recurrence as the response (log-rank p=0.0198); **C)** KM curves comparing the low *HERC5* mRNA expression group (n=62) to the high *HERC5* mRNA expression group (n=180) with overall survival as the response (log-rank p=0.0063). In addition to the KM test of equality, a multivariate Cox PH regression model was also used for both models adjusting for age, cirrhosis status, gender, HBV/HCV status, and TNM staging. Data were taken from Roessler *et al.*, Cancer Res 70(24):10202-12 (2010). **D)** Gene expression $\log_2$ intensity distribution for tumor biopsies from HCC patients (green), where the blue line indicates the median $\log_2$ intensity across 65 patients; **E)** KM curves comparing the low *HERC5* mRNA expression group (n=20) to the high *HERC5* mRNA expression group (n=24) with progression-free survival as the response (log-rank p=0.067); **F)** KM curves comparing the low *HERC5* mRNA expression group (n=20) to the high *HERC5* mRNA expression group (n=24) with overall survival as the response (log-rank p=0.023). In addition to the KM test of equality, a multivariate Cox PH regression model was used for both models adjusting for age,

gender, and HBV status. Data were taken from *Boyault et al.*, Hepatology 45(1):42-52 (2007).

[034] Figure 4 shows WES somatic SNV pattern in the primary and recurrent tumors of four HCC patients.

[035] Figure 5 shows SNVs shared between primary and recurrent tumors show clonal advantage compared to those unique to primary tumors in the 4 HCC patients. Error bars represent 95% confidence intervals.

[036] Figures 6A-D shows non-silent somatic SNV Vfs between the recurrent tumor (y-axis) and primary tumor (x-axis) and barplots showing the proportion of counts unique to the primary tumor (salmon), shared between the primary and recurrent tumors (light blue), or unique to the recurrent tumor (green) for: HCC1, HCC4, HCC5, and HCC11.

[037] Figure 7 provides distribution of transformed p-values for *HERC5* high/low group from Cox PH regression model predicting HCC recurrence for genes within chromosome 4q deletion (x-axis is physical coordinates) using the *Roessler et al.* study.

[038] Figures 8A-B illustrates the expression profile of *HERC5* correlating with 186-gene signature used to predict prognosis (good=red; green=intermediate; light blue=poor) of patients with HCV-positive cirrhosis in liver specimens. **A)** Distribution of 3 prognoses levels determined from Hoshida et al, study (good: n=60; intermediate: n=101; poor: n=55). **B)** Distribution of 2 prognoses levels determined from *Hoshida et al.*, Gastroenterology 144:1024-30 (2013) (good: n=109; poor: n=107). Table indicates risks calculated from time-to-event analyses using the 186-gene signature.

[039] Figures 9A-B provide **A)** a WES DNA germline SNP correlation heatmap of 5 specimens (primary tumor, recipient normal adjacent tissue, recurrent tumor, donor normal adjacent tissue, and recipient blood) per patient using 300 heterozygous SNPs identified from the 1000 genomes project database; and **B)** mRNA correlation heatmap for the same patients,

without the recipient blood specimens. Brown/yellow/white blocks and cluster dendrograms on each axis indicate similarity in groupings of each specimen quintet for DNA and quartet for mRNA.

[040] Figure 10 illustrates a WES DNA germline SNVs heatmap of 5 specimens (primary tumor, recipient normal adjacent tissue, recurrent tumor, donor normal adjacent tissue, and recipient blood) per patient.

[041] Figure 11 demonstrates determination of tumor origin using a derivation of the clonal relationship value calculation and all non-silent SNVs with medium-to-high polyphen predictions.

[042] Figures 12A-D provides donor contamination calculations using the slope (m) of all somatic SNV Vfs between the recurrent tumor (y-axis) and primary tumor (x-axis) for HCC1, HCC4, HCC5, and HCC11. All somatic variants unique to either primary tumor or recurrent tumor have been removed from the plots, as have any $Vf < 5\%$ in either the primary or recurrent tumor samples. Red dashed line indicates $x=y$ and solid blue line indicates the least squares fit.

[043] Figures 13A-C illustrates donor contamination B-allele frequency (BAF) histograms using recipient-unique homozygous single nucleotide polymorphisms (SNPs) for HCC1, HCC4, and HCC5. After removal of all alleles affected by CNVs, the contamination of donor cells was calculated as the median($1 - Vf$ s of the recipient-unique homozygous alleles in the recurrent tumor). Estimates of donor tissue contamination are as follows: HCC1=72%, HCC4=3%, and HCC5=48%. HCC11 primary and recurrent tumors are of recipient-origin, so that sample is not included here.

DESCRIPTION OF THE SEQUENCES

[044] Table 1 provides a listing of certain sequences referenced in present embodiments.

Table 1: Table of the Sequences		
Description		SEQ ID NO

HERC5 Sequence	>gi 380420368 ref NM_016323.3 Homo sapiens HECT and RLD domain containing E3 ubiquitin protein ligase 5 (HERC5), mRNA TCAGTAGCTGAGGCTGCGGTTCCCCGACGCCACGCAGCTGCGCGCAGCTGGTTC CCGCTCTGCAGCGCAACGCCTGAGGCAGTGGGCGCGCTCAGTCCCGGGACCAGG CGTTCTCTCCTCTCGCCTCTGGGCCTGGGACC CCGCAAAGCGGCGATGGAGCGGAGGTCGCGGAGGAAGTCGCGGCGCAACGGGCG CTCGACCGCGGGCAAG GCCGCCGCGACCCAGCCCGGAAGTCTCCGGGCGCACAGCTCTGGCTCTTTCCC AGCGCCGCGGGCCTCC ACCGCGCGCTGCTCCGGAGGGTGGAGGTGACGCGCCAACTCTGCTGCTCGCCGG GGCGCCTCGCGGTCTT GGAACGCGGGCGGGCGGGCGTCCAGGTTACCAGCTGCTCGCCGGGAGCGGCGG CGCCCGGACGCCGAAA TGCATTAAATTAGGAAAAAACATGAAGATACATTCCGTGGACCAAGGAGCAGAG CACATGCTGATTCTCT CATCAGATGGAAAACCATTTGAGTATGACAACTATAGCATGAAACATCTAAGGT TTGAAAGCATTTTACA AGAAAAAAAATAATTCAGATCACATGTGGAGATTACCATTCTCTTGCACTCTC AAAAGGTGGTGAGCTT TTTGCTTGGGGACAGAACCTGCATGGGCAGCTTGGAGTTGGAAGGAAATTTCCC TCAACCACCACACCAC AGATTGTGGAGCACCTCGCAGGAGTACCCTTGGCTCAGATTTCTGCCGGAGAAG CCCACAGCATGGCCTT ATCCATGTCTGGCAACATTTATTTCATGGGGAAAAAATGAATGTGGACAACTAGG CCTGGGCCACACTGAG AGTAAAGATGATCCATCCCTTATTGAAGGACTAGACAATCAGAAAGTTGAATTT GTCGCTTGTGGTGGCT CTCACAGTGGCCTACTCACACAGGATGGGCTGCTGTTTACTTTCCGGTGCTGGAA AACATGGGCAACTTGG TCATAATTCAACACAGAATGAGCTAAGACCCTGTTTGGTGGCTGAGCTTGTGG GTATAGAGTGACTCAG ATAGCATGTGGAAGGTGGCACACACTTGCCATGTTTCTGATTTGGGAAAGGTC TTTTCCTTTGGTTCTG GAAAAGATGGACAACCTGGGAAATGGTGGAACACGTGACCAGCTGATGCCGCTTC CAGTGAAAGTATCATC AAGTGAAGAACTCAAACCTTGAAGCCATACCTCAGAAAAGGAGTTAATAATGAT TGCTGGAGGGAATCAA AGCATTTTGTCTCTGGATAAAAGAAAGAGAATTCATATGTTAATCTGAAGAGGACA ATTCCTACTCTGAATG AAGGGACTGTAAAGAGATGGATTGCTGATGTGGAGACTAAACGGTGGCAGAGCA CAAAAAGGGAAATCCA AGAGATATTTTCATCTCCTGCTTGTCTAACTGGAAGTTTTTTAAGGAAAAGAAG AACTACAGAAATGATG CCTGTTTATTTGGACTTAAATAAAGCAAGAAACATCTTCAAGGAGTTAACCCAA AAGGACTGGATTACTA ACATGATAACCACCTGCCTCAAAGATAATCTGCTCAAAGACTTCCATTTTCATT CTCCACCCCAAGAAGC TTTAGAAATTTTCTTCTTCTCCAGAATGTCCTATGATGCATATTTCCAACAA CTGGGAGAGCCTTGTG GTTCCATTTGCAAAGGTTGTTTGTAATAAGAGTGACCAGTCTTCACTGGTTCCTG GAAGAGTATTGGGCAA CTCTGCAAGAATCCACTTTTCAGCAAACCTGGTCCAGATGTTTAAAACAGCCGTCA TATGCCAGTTGGATTA CTGGGATGAAAGTGCTGAGGAGAATGGTAATGTTCAAGCTCTCCTAGAAATGTT GAAGAAGCTGCACAGG GTAAACCAGGTGAAATGTCAACTACCTGAAAGTATTTTCCAAGTAGACGAACTC TTGCACCGTCTCAATT TTTTTGTAGAAGTATGCAGAAAGTACTTGTGGAAAATGACTGTGGACGCTTCAG AAAATGTACAATGCTG CGTCATATTCAGTCACTTTCCATTTATCTTTAATAATCTGTGAAAAATTAACT ACTACATACAGACACA	1
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DESCRIPTION OF THE EMBODIMENTS

I. Methods of Treating Hepatocellular Carcinoma

A. Evaluating HERC5 mRNA Expression

[045] Hepatocellular Carcinoma (HCC) may be treated through an orthotopic liver transplant (OLT). As recurrence of the primary tumor negatively impacts the success rate for OLT, even after using clinical criteria to qualify patients for OLT, there is a need for additional information to assist a physician in selecting appropriate candidates for OLT, specifically those with a lower risk of recurrence.

[046] In one embodiment, a method of treating hepatocellular carcinoma in a patient comprises obtaining a hepatocellular carcinoma sample from the patient; determining whether the sample has a normal level of HERC5 mRNA expression; and qualifying the patient for an orthotopic liver transplant if the sample has a normal level of HERC5 mRNA expression. In one embodiment, the hepatocellular carcinoma sample has a HERC5 DNA deletion and the patient is not qualified for an orthotopic liver transplant. In another embodiment, the hepatocellular carcinoma sample has HERC5 mRNA underexpression and the patient is not qualified for an orthotopic liver transplant. In another embodiment, a patient with a normal level of HERC5 mRNA is given priority on a transplant waiting list over at least one hepatocellular carcinoma patient without a normal level of HERC5 mRNA. In another embodiment, a patient with a normal level of HERC5 mRNA is given priority on a transplant waiting list over any hepatocellular carcinoma patient without a normal level of HERC5 mRNA. In a further embodiment, the patient with a normal level of HERC5 mRNA receives a liver transplant.

[047] In one embodiment, the patient does not qualify for sorafenib. In another embodiment, the patient was treated with sorafenib and the treatment was unsuccessful.

B. Measuring HERC5 mRNA Levels

1. Obtaining an *Ex Vivo* Sample

[048] In some embodiments, in order to measure the level of HERC5 mRNA, a tumor sample is removed from the patient. In one embodiment, this step is performed on a biopsy sample that is removed for other purposes, such as to diagnose HCC or stage the tumor. The biopsy may be removed surgically or it may be removed through a needle biopsy. In one embodiment, techniques may be used that reduce seeding of tumor cells after the biopsy, such as using a coaxial cutting needle technique employing an introducer with a wider diameter (for example, a 17-gauge introducer) and a biopsy needle with a smaller diameter (for example, an 18-gauge biopsy needle) so that the needle introducer remains in position during at least one or more cutting needle passes and protects normal tissue along the track of the biopsy. In one embodiment, needle core biopsy may be employed and in another, fine needle aspiration cytology may be used. In one embodiment, laparoscopic surgery techniques may be used to obtain a biopsy. In other embodiments, ultrasound or other imaging techniques may be used to assist in obtaining the sample.

2. Determining HERC5 mRNA Levels in an *Ex Vivo* Sample

[049] A variety of standard molecular biology techniques can be used to determine HERC5 levels in an *ex vivo* sample and the present embodiments are not limited to any mode or method of detecting HERC5 levels in a sample. In one mode, mRNA levels may be detected. In another mode, DNA levels may be detected. In one embodiment, the patient has a single hepatocellular carcinoma lesion. In another embodiment, the patient has multiple hepatocellular carcinoma lesions. In the embodiment where the patient has multiple lesions, the HERC5 levels may be determined in one, some, or all of the lesions.

[050] In one mode, the level of HERC5 mRNA may be detected in a sample using a microarray, such as a DNA or mRNA microarray. In a DNA or mRNA microarray, a probe

that binds to the HERC5 sequence of interest may be bound through surface engineering to the solid surface of the microarray by a covalent bond. The solid surface may be a glass or silicon chip or it may include microscopic beads. Probe-target hybridization may be detected and quantified by using fluorophore-, silver-, or chemiluminescence-labeled targets to determine the relative abundance of nucleic acid sequences in the target.

[051] A variety of commercially-available DNA or mRNA microarrays may be employed in this embodiment, such as, but not limited to, an Affymetrix[®] chip or Illumina[®] BeadArray microarray technology.

[052] In another mode, the level of HERC5 mRNA may be detected in a sample using PCR, for example quantitative PCR (qPCR) also known as real-time PCR. qPCR enables both detection and quantification, which can be an absolute number of copies of a sequence or a relative amount when normalized to a DNA input. mRNA-based PCR may also be used by first reverse-transcribing the mRNA sequence into cDNA with a reverse transcriptase (reverse transcriptase quantitative PCR or RT-qPCR).

[053] The amplified DNA (either from a DNA sample or cDNA reverse transcribed from mRNA) may be detected as the reaction progresses in real time, through one or more approaches, including but not limited to using nonspecific fluorescent dyes that intercalate with any double-stranded DNA and/or sequence-specific DNA probes (such as oligonucleotides) that are labelled with a fluorescent reporter which permits detection after hybridization of the probe with its complementary sequence. In the nonspecific mode, a DNA-binding dye, such as SYBR Green, will bind to all dsDNA PCR products and in this mode the only sequence-specific reagents required are the primers for amplification of HERC5. In the sequence-specific DNA probe mode, a fluorescent reporter detects only the DNA containing the probe sequence, increasing specificity and enabling quantification even in the presence of nonspecific DNA amplification. In some modes, the sequence-specific

DNA probe has a fluorescent reporter on one end and a quencher on the other end to prevent fluorescence unless it is bound to the target DNA strand.

[054] A variety of commercially-available qPCR kits can be employed in this embodiment, such as, but not limited to, GoTaq[®] dye-based qPCR and RT-qPCR, GoTaq[®] probe-based qPCR and RT-qPCR, and Plexor[®] qPCR and RT-qPCR systems, all from Promega[®].

C. Comparing HERC5 mRNA Expression

1. Control Populations and Techniques for Comparison

[055] Once the HERC5 level has been quantified, it may be evaluated against a cutoff level or a control. A cutoff level may be obtained by performing a sufficient number of experiments in a control group. Thus, a control may be a side-by-side control experiment or it may be a historical control. In one embodiment, the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a historical control group. In one embodiment, the control is a healthy liver tissue sample or historical data from evaluation of healthy liver tissue samples.

[056] In one embodiment, the historical control group has normal subjects. In another embodiment, the historical control group has patients who had a hepatocellular carcinoma that did not recur within at least 5, 6, 7, 8, 9, or 10 years. These control groups may be referred to as negative control groups demonstrating “normal” levels, in that they either had no hepatocellular carcinoma or in that they had a hepatocellular carcinoma that did not recur within a designated period of time. In one embodiment, the HERC5 patient sample level is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical negative control group. In another embodiment, HERC5 patient sample is designated as normal if it is equal to or higher than one standard deviation below the mean of the historical negative control group.

[057] In a further embodiment, the historical control group has patients who had a hepatocellular carcinoma that recurred within 1, 2, 3, 4, or 5 years. This control group may be referred to as a positive control group demonstrating “low” levels, in that they had a hepatocellular carcinoma recurrence within a designated period of time. In one mode, HERC5 patient sample level is designated as normal if it is equal to or higher than one standard deviation above the mean of the positive control group.

[058] In yet a further embodiment, the historical control group comprises patients who have had hepatocellular carcinoma, without differentiating between those who have had or not had recurrences. This historical control group may be referred to as an all-HCC control group. In one embodiment, the HERC5 patient sample level is designated as normal if it is equal to or higher than one standard deviation above the mean of the all-HCC control group. In another embodiment, the HERC5 patient sample is designated as normal if it is higher than the median of the all-HCC control group.

[059] In any of these modes, historical control group may comprise data from at least 10, 25, 50, 100, 250, 500, or 1000 individuals. In one embodiment, more than one technique or control may be employed.

2. Healthy Tissue Controls and Techniques for Comparison

[060] In another mode, healthy tissue may be obtained from a patient with a hepatocellular carcinoma and the mRNA level of HERC5 in the healthy tissue may be compared to the mRNA level of HERC5 in hepatocellular carcinoma sample. In one embodiment, the normal tissue is liver tissue.

[061] In one aspect, if the mRNA level of HERC5 in the hepatocellular carcinoma sample higher than 70%, 75%, 80%, 85%, 90% of the level in the normal tissue sample, the HERC5 level is provided as a positive factor indicating that the patient qualifies for an orthotopic liver transplant. In some circumstances, additional clinical factors are also used.

[062] In another aspect, when the mRNA level of HERC5 in the hepatocellular carcinoma sample is lower than 40%, 45%, 50%, 55%, 60%, or 65% of the level in the normal tissue sample, the HERC5 level is provided as a negative factor against the patient qualifying for an orthotopic liver transplant.

D. Inclusion of Clinical Criteria

[063] In any of the modes described herein, a further embodiment may include taking into account clinical criteria in the decision-making process for whether to qualify or disqualify a patient for orthotopic liver transplant. In one embodiment, the clinical criteria comprise at least one of the size of the lesion, the number of lesions, whether there are extrahepatic manifestations, whether there is vascular invasion, whether the HCC was poorly differentiated on biopsy, tumor state, tumor grade, and alpha-fetoprotein levels.

[064] For example, in one aspect, the clinical criteria comprise the Milan criteria: (a) a single lesion not exceeding 5 cm or no more than 3 lesions, none greater than 3 cm; and (b) no macrovascular invasion. In another aspect, the clinical criteria comprise the UCSF criteria: (a) a single lesion not greater than 6.5 cm; or (b) no more than 3 lesions, none of which exceed 4.5 cm and whose total tumor diameter does not exceed 8 cm.

In a further aspect, the clinical criteria comprise the Toronto criteria and include any tumor size or number and no systemic symptoms as a result of HCC and histological-based exclusion of poorly differentiated HCC (beyond Milan tumors only).

[065] In a further aspect, the clinical criteria comprise the Hangzhou criteria and include a) total tumor diameter less than or equal to 8 cm; (b) total tumor diameter more than 8 cm, with histopathologic grade I or II and preoperative AFP level less than or equal to 400 ng/mL.

[066] In one mode, the criteria also include whether the α -fetoprotein concentration is less than 400 ng/mL (with a lower level indicating a potential positive outcome after OLT).

II. Method of Evaluating the Recurrence Risk for a Hepatocellular Carcinoma

[067] In another aspect, the embodiments include a method of evaluating the recurrence risk profile of a hepatocellular carcinoma comprising:

- a. providing a hepatocellular carcinoma sample;
- b. determining *in vitro* whether the sample has a normal level of HERC5 mRNA expression;
- c. qualifying the hepatocellular carcinoma as having a low risk of recurrence if the sample has a normal level of HERC5 mRNA expression and the determination is made in conjunction with at least one clinical criteria.

[068] In one embodiment, the patient does not qualify for sorafenib. In another embodiment, the patient was treated with sorafenib and the treatment was unsuccessful.

[069] One embodiment encompasses the use of a HERC5 forward and reverse primer, each of which is generated from mRAN sequence of HERC5, for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant. Another embodiment encompasses the use of a HERC5-specific probe for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant. Another aspect encompasses the use of a HERC5 nucleic acid sequence as a quantitative control for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant. In either embodiment, the diagnostic agent may be coupled with other clinical criteria. In another embodiment, the diagnosticum is used to

evaluate the recurrence risk for hepatocellular carcinoma after an initial diagnosis has been made and to assist in determining a treatment plan.

[070] The same techniques for evaluating HERC5 levels, comparing HERC5 levels, and incorporating clinical criteria may be used as in section I.B through I.D above.

[071] Reference will now be made in detail to the present exemplary embodiments, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts. Other embodiments will be apparent to those skilled in the art from consideration of the specification and practice disclosed herein. The embodiments are further explained in the following examples. These examples do not limit the scope of the claims, but merely serve to clarify certain embodiments. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit being indicated by the following claims.

EXAMPLES

Example 1. DNA sequence read mapping and variant calling

A) Whole exome and RNA sequencing

[072] Whole exome and RNA sequencing (WES and RNASeq) were conducted with primary and recurrent tumors (P_T and R_T), normal adjacent tissues (P_{NAT} and R_{NAT}), and recipient blood (P_B) from four Chinese HCC OLT patients, with whole genome sequencing conducted on the primary specimens (Table 2A (recurrent patients) and Table 2B (nonrecurrent patients)). All four patients were within the UCSF criteria, two tested positive for Hepatitis B virus at time of OLT; three patients had recurrent liver tumors and one patient had a lung metastasis within 24 months following OLT. DNA whole genome sequence (WGS) and whole exome sequence (WES) data was generated using the Illumina standard library preparation and sequencing protocols. Paired end FASTQ files of 90mer sequence reads for both sequence data types were provided.

[073] All sequence data was QCd for read counts, quality values, kmer usage, GC-content, and all other relevant parameters within FastQC (v0.10.1). The DNA sequence was aligned to the human genome (UCSC hg19; Feb 2009 release; Genome Reference Consortium GRCh37) using Bowtie2 (v2.0.0-beta7; *Langmead et al.*, Nat Methods 9(4):2357-9 (2012)) and both indel realignment and PCR duplicate removal was conducted using GATK (v2.3.4; *McKenna et al.*, Genome Res 20:1297-303 (2010)) and Picard (v1.85), respectively. All mapping summaries for each specimen from WES are provided in **Table 3**. For RNASeq and WES data, SAMtools (v0.1.18; *Li et al.*, Bioinformatics 25:2078-9 (2009)) mpileup was used (Qphred>30 and mapping quality>30) with VarScan2 (v2.3.2; *Koboldt et al.*, Genome Res 22(3):568-76 (2012)) to call both single nucleotide variants (SNVs) and insertion/deletions (indels) relative to the human reference with parameters: variant minimum frequency>5%, VarScan2 p-value for variant calls (based on Fisher's exact test) <0.01, minimum coverage=20, minimum reads=3, minimum average quality=30, minimum frequency for homozygote=0.8. For WGS data, where depth was much lower than WES data, the following arguments were used: minimum coverage=15, minimum reads=2, minimum average quality=13, Qphred>13 and mapping quality>1. Mean WES depth for the 20 specimens ranged from 66-109X (mean=92.1X) with an average of 73.4% coverage (Qphred>30) at a minimum of 10X depth (Table 3).

Table 2A: Patient Clinical Summary (Recurrent Patients)										
Patient ID	HCC1	HCC4	HCC5	HCC11	HCC2	HCC3	HCC6	HCC8	HCC10	
Gender	M	M	M	F	M	M	M	M	M	
Age	48	59	67	57	43	42	41	42	59	
HBV (pre-OLT)	Positive	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Positive	
HBV (post-OLT)	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	
Biopsy information										
Primary tumor and NAT mRNA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Primary tumor biopsy method	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	
Primary tumor content (%)	90	90	70	90	60	80	40	90	80	
Primary tumor size (cm)	5	8	5	2.5	9	6	7	10	4	
Primary tumor grade	III	II	II	II-III	II	III	III	III	III	
Primary tumor cirrhosis	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	
Recurrent tumor method	RFA	RFA	RFA	VATS	RFA	RFA	VATS	RFA	RFA	
Recurrent tumor organ	liver	liver	liver	lung	liver	liver	lung	liver	liver	
Recurrent tumor size (cm)	2	2.5	3	2.5	NA	1.5	NA	1.2	1	
Recurrent tumor content (%)	NA	NA	60	70	NA	NA	70	NA	NA	
Recurrent tumor grade	NA	NA	NA	II-III	NA	NA	NA	NA	NA	
Recurrent tumor cirrhosis	No	No	NA	NA	NA	Yes	NA	NA	No	

Table 2A: Patient Clinical Summary (Recurrent Patients) (continued)										
Primary tumor liver enzymes										
Patient ID	HCC1	HCC4	HCC5	HCC11	HCC2	HCC3	HCC6	HCC8	HCC10	
Glu	5.19	5.4	8.03	13.79	5.42	7.23	4.48	5.1	5.03	
ALT	37	20	54	45	19	30	28	87	71	
AST	39	19	64	54	33	56	30	65	96	
GGT	133	37.7	213.2	120.8	123.4	198.7	39.7	380.2	79.5	
TB	22.8	17.4	56.7	32.6	20.4	34.8	10	22.2	29.6	
DB	5.7	3.9	22.9	9.2	6.7	15.5	4.2	5.8	10.7	
ALB	40.6	43.7	30.2	30.8	41.6	33.9	47.4	39.3	40.2	
Hgb	131	145	112	80	140	144	114	139	136	
PT	12.1	11.6	12.9	14.8	11.9	12.3	10.8	12.1	12.7	
HBsAg	>240(+)	15.22(+)	<0.01(-)	0.03(-)	>240(+)	>240(+)	>250(+)	>250(+)	>250(+)	
HBVDNA	<1000	<500	<500	<500	6.21E+03	<500	<500	<500	<500	
AFP	2935.9	2.5	>3000	1581.6	1461.1	>3000	126	>3000	1888.2	
CEA	2.07	1.27	2.34	1.23	4.41	3.3	NA	1.85	3.97	
CA199	33.2	8.4	28.7	51.8	<0.8	119.8	NA	44.8	70.8	
BCLC Stage pre-LTx	A3	B	B	B	B	B	B	B	A3	
RFA=Radiofrequency ablation; VATS=Video-assisted thorascopic surgery; OLT=Orthotopic liver transplantation; AFP=Alpha-fetoprotein										

Table 2B: Patient Clinical Summary (Nonrecurrent Patients)												
Patient ID	HCC-C2	HCC-C6	HCC-C8	HCC-C9	HCC-C11	HCC-C12	HCC-C13	HCC-C14	HCC-C15	HCC-C16	HCC-C18	HCC-C19
Gender	M	M	M	M	M	M	M	M	M	M	M	M
Age	55	51	64	40	62	40	49	59	49	49	57	41
HBV (pre-OLT)	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
HBV (post-OLT)	Negative	Negative	Positive	NA	Negative		Negative	NA	Negative	NA	Negative	Positive
Biopsy information												
Primary tumor and NAT RNA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Primary tumor biopsy method	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT	OLT
Primary tumor content (%)	90	85	95	80	90	70	90	60	90	80	95	90
Primary tumor size (cm)	5	3	1.5	1	3	2.5	3	7	5	5	5	4
Primary tumor grade	I-II	II	I	II	I	II - III	I-II	II	II	II - III	I	II - III
Primary tumor cirrhosis	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 2B: Patient Clinical Summary (Nonrecurrent Patients) (continued)												
Primary Tumor Liver Enzymes												
Patient ID	HCC-C2	HCC-C6	HCC-C8	HCC-C9	HCC-C11	HCC-C12	HCC-C13	HCC-C14	HCC-C15	HCC-C16	HCC-C18	HCC-C19
Glu	4.55	7.52	4.73	5.32	12.01	NA	4.19	5.46	8.64	4.89	4.95	4.85
ALT	78	11	16	16	27	38	26	197	34	52	43	12
AST	134	14	25	18	34	19	38	296	24	140	38	16
GGT	64.2	5.8	56.4	35.3	75.8	47.3	55	53.6	171.6	68.9	139.9	21.1
TB	35.8	52.1	47.7	19.7	34.5	12.7	22.1	74.7	33.3	64.9	16.3	21.5
DB	14.1	15.5	16.1	5.2	10.3	4	3.5	25.1	10.5	23.2	6.5	7
ALB	22.3	31.6	30.8	42.7	40.5	43.5	25.8	31.4	45.6	30	39.2	46
Hgb	113	75	148	158	137	150	130	118	146	92	150	142
PT	17	16.3	15.7	11.9	12.4	11.2	12.1	15.8	11.5	14.9	13.4	12.9
HBsAg	>240	>240	>240	+	>240	>240	>240	>240	>240	>240	>250	219.91
HBVDNA	1.04E+06	<1000	<1000	1.29E+03	<1000	1.10E+05	<500	9.00E+06	<500	<500	2.01E+04	<500
AFP	5.2	137.5	62.5	9.2	5.4	210.8	4.3	240.7	NA	128.9	6.6	220.9
CEA	4.79	1.4	2.81	4.85	2.6	1.85	1.97	0.87	NA	2.11	1.85	2.01
CA199	72.6	4.5	21	24.6	51.8	21.3	21.5	27.1	NA	29.5	45.6	19
BCLC Stage pre-LTx	D	A3	A3	A3	A3	A2	C	B	B	A3	A2	B

Table 3: WES depth summary for reads with Qphred values>30 and mapping quality values>30 for each specimen													
Subject	Specimen	1st Qu.	Median	Mean	3rd Qu.	Max.	total bases	count >=10	% >=10X	count >=20	% >=20X	count >=50	% >=50X
HCC1-D-13C-1	Recipient blood	4	58	76.39	124	2551	55924905	37951533	67.86%	35761057	63.94%	29741078	53.18%
HCC1-D-13P-1	Primary NAT	9	88	106.5	169	2589	51425740	38349927	74.57%	36584748	71.14%	32227656	62.67%
HCC1-D-13P-12	Recurrent NAT	8	77	93.18	148	1682	51063906	37898098	74.22%	35951622	70.41%	30809365	60.33%
HCC1-D-13T-1	Primary tumor	9	77	100.8	153	7962	51080376	38049758	74.49%	36009742	70.50%	30750062	60.20%
HCC1-D-13T-12	Recurrent tumor	22	90	105.3	161	3808	47812800	38115772	79.72%	36304664	75.93%	31395548	65.66%
HCC11-D-13C-2	Recipient blood	6	87	106.7	172	2088	52344605	38274510	73.12%	36415998	69.57%	32081724	61.29%
HCC11-D-13P-1	Primary NAT	10	87	102.4	162	2255	50611343	38091316	75.26%	36247797	71.62%	31685450	62.61%
HCC11-D-13P-2	Recurrent NAT	8	81	96.75	154	1817	52336091	38695121	73.94%	36785205	70.29%	32008107	61.16%
HCC11-D-13T-1	Primary tumor	9	78	95.96	150	1389	50461219	37746861	74.80%	35791452	70.93%	30594770	60.63%
HCC11-D-13T-2	Recurrent tumor	4	63	86.47	138	1508	56502573	38524822	68.18%	36380292	64.39%	30889148	54.67%
HCC4-D-13C-4	Recipient blood	8	81	98.28	156	1376	52189962	38583465	73.93%	36657013	70.24%	31876256	61.08%
HCC4-D-13P-4	Primary NAT	7	90	109	175	1624	52242043	38505355	73.71%	36714405	70.28%	32527534	62.26%
HCC4-D-13P-15	Recurrent NAT	4	54	72.08	118	2367	56975058	37904212	66.53%	35694718	62.65%	29450212	51.69%
HCC4-D-13T-4	Primary tumor	9	72	92.55	142	1459	51093067	38067394	74.51%	35982067	70.42%	30286699	59.28%
HCC4-D-13T-1	Recurrent tumor	11	51	65.98	98	1098	48425601	36670782	75.73%	33831567	69.86%	24750162	51.11%

15																
HCC5-D-13C-5	Recipient blood	8	73	89.48	141	1500	52050460	38498173	73.96%	36414724	69.96%	30878403				
HCC5-D-13P-5	Primary NAT	9	72	85.82	136	1502	50649256	37766775	74.57%	35705182	70.49%	29969073				59.17%
HCC5-D-13P-16	Recurrent NAT	5	66	81.28	129	2127	52392114	37781565	72.11%	35719272	68.18%	29816702				56.91%
HCC5-D-13T-5	Primary tumor	9	68	83.25	130	1309	51303768	38192411	74.44%	36025209	70.22%	29838848				58.16%
HCC5-D-13T-16	Recurrent tumor	8	77	94.46	148	1790	51566852	38189917	74.06%	36213036	70.23%	31059046				60.23%

[074] We identified 1,145 somatic variants in both P_{TS} and R_{TS} including 616 somatic nonsilent (nonsynonymous, stop-gain, stop-loss, or frameshift substitution) single nucleotide variants (SNVs) or insertion/deletions (indels) affecting 567 genes (Table 4 and data not shown). The somatic SNV distribution in had highest occurrences of C>T/G>A and lowest occurrences of T>G/A>C (Figure 4), consistent with a previous HCC report (*Totoki et al.*, Nat Genet 43:464-71 (2011)), and matched P_T and R_T pairs had similar transition/transversion substitutions distributions, with HCC11 having highest prevalence of T>A/A>T. Nonsilent-to-silent SNV rate averaged 2.9 in P_{TS} and 2.3 in R_{TS} – a lower ratio in R_{TS} a result of donor-tissue contamination We identified 1,145 somatic variants in both P_{TS} and R_{TS} including 616 somatic nonsilent (nonsynonymous, stop-gain, stop-loss, or frameshift substitution) single nucleotide variants (SNVs) or insertion/deletions (indels) affecting 567 genes (Table 4 and data not shown). The somatic SNV distribution in had highest occurrences of C>T/G>A and lowest occurrences of T>G/A>C (Figure 4), consistent with a previous HCC report (*Totoki et al.*, Nat Genet 43:464-71 (2011)), and matched P_T and R_T pairs had similar transition/transversion substitutions distributions, with HCC11 having highest prevalence of T>A/A>T. Nonsilent-to-silent SNV rate averaged 2.9 in P_{TS} and 2.3 in R_{TS} – a lower ratio in R_{TS} a result of donor-tissue contamination.

Table 4: WES somatic SNV and (Indel) count summary					
	HCC1	HCC4	HCC5	HCC11	Average
All					
Primary tumor total (indels)	239 (3)	138 (7)	93 (4)	305 (11)	193.8 (6.3)
Primary tumor unique (indels)	107 (3)	33 (3)	32 (2)	19 (1)	47.8 (2.3)
Recurrent tumor total (indels)	268 (2)	121 (4)	164 (4)	371 (11)	231 (5.3)
Recurrent tumor unique (indels)	136 (2)	16 (0)	103 (2)	85 (1)	85 (1.3)
Primary and Recurrent tumor shared (indels)	132 (0)	105 (4)	61 (2)	286 (10)	146 (4)
Silent					
Primary tumor total (indels)	48 (1)	32 (0)	24 (2)	64 (2)	42
Nonsilent/Silent Primary tumor ratio	3.1	2.8	2.4	3.1	2.9
Primary tumor unique (indels)	22 (1)	9 (0)	11 (1)	4 (0)	11.5
Recurrent tumor total (indels)	67 (1)	27 (0)	38 (1)	75 (2)	51.75
Nonsilent/Silent Recurrent tumor ratio	1.5	2.9	1.8	3.1	2.3
Recurrent tumor unique (indels)	41 (1)	4 (0)	25 (0)	15 (0)	21.25
Primary and Recurrent tumor shared (indels)	26 (0)	23 (0)	13 (1)	60 (2)	30.5
Nonsilent					
Primary tumor total (indels)	147 (2)	88 (7)	58 (2)	199 (9)	123 (5)
Primary tumor unique (indels)	59 (2)	20 (3)	17 (1)	12 (1)	27 (1.8)
Recurrent tumor total (indels)	103 (1)	79 (4)	69 (3)	233 (9)	121 (4.3)
Recurrent tumor unique (indels)	15 (1)	11 (0)	28 (2)	46 (1)	25 (1)
Primary and Recurrent tumor shared (indels)	88 (0)	68 (4)	41 (1)	187 (9)	96 (3.5)
Primary tumor Vf mean/median	35.9/31.1	21.0/16.9	19.9/16.1	16.9/12.6	23.4/19.1
Primary and Recurrent tumor shared Vf mean/median	50.4/46.2	38.0/35.2	32.2/29.2	37.2/36.3	39.4/36.7
Welch's t-test p-value	4.06E-05	7.75E-06	9.14E-04	4.39E-05	
Vf=variant allele frequency					

B) Identification of Somatic SNVs

1. Methods

[075] Variant calls were made for all samples in a batch call and exported as a Variant Call Format (VCF) (v4.1) file. Then variant call refinement was conducted according to the following rules for somatic SNV and indel calls between pairs of P_T vs. P_B , P_{NAT} vs. P_B , R_T vs.

P_B , and R_{NAT} vs. P_B , where P=primary, R=recurrent, T=tumor, NAT=normal adjacent tissue, and B=blood as follows:

- 1) Average Qphred of reference or variant (or both) >30 for WES (13 for WGS) in normal and tumor,
- 2) P-value from Fisher's exact test of read counts between normal/tumor and reference/variant <0.01,
- 3) Variant frequency (Vf) in normal <3%, and
- 4) Tumor Vf at least 5% greater than Vf of normal.

[076] To identify somatic SNVs specific to both the P_T and R_T samples for each patient, those SNVs identified between P_{NAT} vs. P_B that were shared with the list of P_T vs. P_B SNVs were removed, as were those SNVs identified between R_{NAT} vs. P_B that were shared with the list of R_T vs. P_B SNVs. Annotation and variant effects on genes was then conducted using ANNOVAR (v2013-07-28; Wang *et al.*, Nucleic Acids Res 38(16):e164 (2010)) and all nonsilent (i.e. nonsynonymous, stopgain, or stoploss) SNVs or nonsilent indels (i.e. frameshift substitution) were selected based on:

- 1) Either an unknown or minor allele frequency (MAF) <0.05 in the 1000 genomes with all races, and
- 2) Not located within either tandem repeat or disruptive repeat regions from the UCSC Genome database (<http://genome.ucsc.edu>).

[077] All silent SNVs and indels were selected based on predicted exon function (synonymous) and criterion #2 above. Somatic SNV and indel counts for each patient are provided in Table 4.

[078] Germline SNV calls were also refined for the pairs described above according to the following rules:

- 1) Average Qphred of reference or variant (or both) >30 for WES (13 for WGS) in normal and tumor,
- 2) P-value from VarScan2 Fisher exact test (relative to reference genome) <0.01 in both normal and tumor, and
- 3) Minimum Vf in both normal and tumor > 5%.

[079] All nonsilent (i.e. nonsynonymous, stopgain, or stoploss) SNVs or nonsilent indels (i.e. frameshift substitution) were selected based on:

- 1) Either an unknown or minor allele frequency (MAF)<0.05 in the 1000 genomes with all races, and
- 2) Not located within either tandem repeat or disruptive repeat regions from the UCSC Genome database (<http://genome.ucsc.edu>).

[080] This provided the germline SNV list.

2. Results

[081] Clones harboring driver mutations have selection advantage as the major clones in tumors. We evaluated variant frequency (Vf) distributions among all nonsilent SNVs and indels for each patient between loci unique to the P_{TS} and those shared between P_{TS} and R_{TS} (Figure 5). Vfs for variants shared between the P_{TS} and R_{TS} (mean=39.4) were significantly higher than those unique to the P_{TS} (mean=23.4; p<0.001 all four patients; Table 4). Nonsilent variants unique to the P_T, R_T, or shared were also compared among the patients, indicating a large increase in the proportion of clones shared compared to each unique set (Figure 6). This suggests shared somatic mutations to likely be driver mutations.

[082] Somatic CNVs were also identified within P_{TS} and R_{TS} (data not shown) Significantly amplified or deleted regions were selected for uniqueness to the P_T or the R_T, or shared between the two, all within at least 3 of 4 patients (Figure 1). DNA amplifications shared between P_{TS} and R_{TS} were identified, with those marked with an asterisk (*) observed in a previous report (*Guichard et al.*, Nat Genetics 44:694-699 (2012)) in the P_{TS} of HCC patients: 1q*, 6p*, 8q*, 17q*, and 20p*, while shared DNA deletions included: 4q* and 17p*. Amplified regions unique to the R_T were identified within 17q and 20q.

Example 2. RNA sequence read mapping and differential expression analysis

[083] RNA whole transcriptome sequence data was generated by BGI using the Illumina standard library preparation and sequencing protocols. Paired end FASTQ files of 90mer sequence reads were provided to MedImmune. Sequence data was QCd for read counts, quality values, kmer usage, GC-content, and all other relevant parameters within FastQC (v0.10.1). The average read count per mate was 50 million. RNA reads were mapped to the human genome (UCSC hg19; Feb 2009 release; Genome Reference Consortium GRCh37) using TopHat2 (v2.0.9; *Kim et al.*, Genome Biol 14(4):R36 (2013)) and the human reference gtf annotation file (GRCh37.68). Transcript counts were calculated and normalized using htseq-count and DESeq (v1.12.1; *Anders et al.*, Genom Biol 11:R106 (2010)). The DESeq negative binomial distribution was used to calculate the p-value and fold changes between P_T and P_{NAT} as well as R_T and R_{NAT} using $p < 0.01$ and $|\text{fold change}| > 2$ as a threshold.

Example 3. Somatic copy number variation (CNV) analysis

[084] R packages DNACopy and ExomeCNV were used to identify CNVs based on the read depth derived from the WES alignments. A 1 Mb window size was selected to identify CN segments based on three reasons: 1) to identify large-scale segments rather than regions of focal variation, 2) coding region gene structure can be hundreds of kilobases long in exome capture, requiring a large window to encompass entire genes, rather than parts of a gene, and 3) a summary across all patients showed that <3% of segments were excluded at a 1Mb threshold. Log₂ R ratios (LRR) of CNVs were calculated across the genome as the difference between all tumor and normal comparisons (see description above for relevant pairings). Tumoral purity was evaluated using the ABSOLUTE software (*Carter et al*, Nature Biotechnol 30(5):413-21 (2012)) and compared against the pathology assessments for each tumor. All CNV results are provided in Figure 1 (raw data not shown).

Example 4. Pathway analysis**A) Methods**

[085] To further identify key somatic genetic changes, an integrated pathway analysis was employed. Pathway analyses were conducted using Ingenuity Pathway Analysis (IPA; Ingenuity, Redwood City, CA), while plots parsing scripts, and additional analyses were conducted in R (v3.0.1; R: A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2013). Two types of pathway analyses outputs were conducted. The first was calculated to associate the upstream genetic effects on pathway regulation. For this approach, all genes harboring nonsilent somatic SNVs and indels shared between the primary and recurrent tumors for an individual patient with medium-to-high PolyPhen functional effects were combined with genes within CNV regions of significant amplification or deletion AND gene expression $|\text{fold changes}| > 2$ (P_T/P_{NAT} and R_T/NAT_T); p-values from DESeq < 0.05 . These genes were then used in canonical pathway enrichment analysis using IPA. The comparison tool in IPA was used to combine all pathway information from each of the four patients and a composite score was computed per pathway as the summation of the $-\log_{10}(pvs)$ across the patients for a pathway (data not shown). This same pathway enrichment analysis was also conducted for those genes using this same criteria for just those instances unique to the recurrent tumor across all four patients (data not shown). The second pathway analysis output included the upstream analysis output from IPA and differentially expressed transcripts (shared between P_T vs. P_{NAT} and R_T vs. R_{NAT}), which identified those transcription factors (TFs) most enriched by genes that are regulated by the TF (as determined by the literature). This is referred to as an upstream gene signature (Table 5).

B) Results

[086] Nonsilent SNVs and indels with medium-to-high PolyPhen scores and genes within significantly amplified or deleted CN regions, which were also over- or under-expressed ($|\text{fold}| > 2$; $p < 0.05$), and shared between the P_{TS} and R_{TS} ($P_T/P_{NAT} \cap R_T/R_{NAT}$) for each patient were identified. These genes were combined for each patient individually and analyzed for pathways enriched by these factors (data not shown) Transcription factors enriched by differentially expressed transcript targets (denoted as *upstream gene signatures*) for shared tumor regulated genes ($P_T/P_{NAT} \cap R_T/R_{NAT}$) for each patient ($p < 0.01$ and $|\text{fold}| > 2$ were also identified; Table 4).

Table 5: Upstream gene signatures using differentially expressed genes between tumor and normal data as input into IPA (shared between primary and recurrent tumors)

Sample	Shared or Recurrent	Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset	Mechanistic Network
hcc1 - 2013-11-11	shared	CTNNB1	transcription regulator	Activated	2.408	4.10E-03	AFP, CDKN2A, CIDEC, DKK1, ELOVL3, IGF2BP1, KIFC1, S100A8, SFN	CTNNB1, TCF4
hcc4 - 2013-11-11	shared	E2F1	transcription regulator	Activated	2.173	7.84E-04	CCNE2, CDC6, CDKN2A, E2F1, HELLS, IGF1, KIF23, MCM2, MT1G, MYBL2, TOP2A, TP73	E2F1, E2F2, E2F3, E2F4, E2F5, E2F, MYBL2, RB1, RBL1, Rb, TFD P1, TP53
hcc5 - 2013-11-11	shared	E2F1	transcription regulator	Activated	2.194	1.33E-03	CCNB1, CDKN2A, E2F1, MCM10, RRM2, TOP2A	CARM1, E2F1, E2F2, E2F3, E2F, HDAC1, MDM2, RB1, RBL1, S MARCA2, TBX2, TFD P1
hcc1 - 2013-11-11	shared	E2F1	transcription regulator	Activated	2.401	1.49E-02	CDK1, CDKN2A, E2F1, MYBL2, MYCN, TOP2A	
hcc1 - 2013-11-11	shared	FOXO1	transcription regulator	Activated	2.219	6.13E-03	ANLN, ASPM, CDK1, CENPF, DEPDC1, SQLE	CDKN1A, E2F, FOXO1, Rb, TP53
hcc4 - 2013-11-11	shared	FOXO1	transcription regulator	Activated	2	2.40E-01	ANLN, AQP9, ASPM, IGF1, PO U5F1	
hcc5 - 2013-11-11	shared	MYC	transcription regulator	Activated	3.083	2.87E-05	ACSL4, BUB1B, CCNB1, CDC20, CDH1, CDKN2A, E2F1, GPC3, MKI67, RRM2, UBE2C, ZIC2	E2F1, EP300, MAX, MYC, RBL2
hcc4 - 2013-11-11	shared	NKX2-3	transcription regulator	Activated	2	2.23E-01	ANLN, FCN3, MT1M, PLEKHA4	
hcc4 - 2013-11-11	shared	TBX2	transcription regulator	Activated	2.056	1.58E-08	ANLN, ASF1B, CDC6, CDKN2A, E2F1, E2F7, E2F8, HELLS, MCM2, NCAPG2	CDKN1A, E2F, RBL1, Rb, TBX2, TP53
hcc1 - 2013-11-11	shared	TCF4	transcription regulator	Activated	2	2.08E-04	CDKN2A, DKK1, IGF2BP1, PH EX	CTNNB1, TCF4
hcc5 - 2013-11-11	shared	CDKN2A	transcription regulator	Inhibited	-2.449	7.29E-06	CDCA5, CDKN2A, E2F1, IGF2BP3, MKI67, RECQL4, RRM2	CDKN2A, E2F1, E2F, HDAC1, RB1, RBL1, SMARCA2, TBX2
hcc1 - 2013-11-11	shared	CDKN2A	transcription	Inhibited	-2.609	2.41E-05	CDK1, CDKN2A, E2F1, IGF2B	CDKN2A, E2F4, E2F5, E2F, IL6,

2013-11-11			regulator				P3,KIFC1,MKI67,MYBL2,MYCN	MYBL2,MYC,RBL1,RBL2,Rb,TFDP1
hcc4 - 2013-11-11	shared	CDKN2A	transcription regulator	Inhibited	-2.985	1.48E-04	ASF1B,CDKN2A,DDR1,E2F1,IGF2BP3,KIFC1,MELK,MKI67,MYBL2,TCF19	CDKN2A,E2F1,E2F4,E2F5,E2F6,EP300,MYBL2,RB1,RBL1,RBL2,Rb,TFDP1,TP53
hcc4 - 2013-11-11	shared	HIF1A	transcription regulator	Inhibited	-2.209	1.42E-01	AQP9,CDKN2A,DTX1,IGF2,IGFBP2,KRT19	
hcc4 - 2013-11-11	shared	HNF4A	transcription regulator	Inhibited	-2.563	1.20E-02	AASS,ABC11,AGXT2,ALDH8A1,AQP9,CCL16,CCNE2,CD6,CDHR2,CYP1A1,CYP1A2,CYP8B1,DTL,F9,FETUB,GLYAT,GYS2,HSD11B1,IGF1,MAPK8IP2,MBL2,MT1H,MT1X,NPC1L1,NUF2,PEMT,POU5F1,PROZ,SERPINA4,SHBG,SLC10A1,TCF19,XPNP	
hcc5 - 2013-11-11	shared	KDM5B	transcription regulator	Inhibited	-2.213	5.31E-05	BUB1B,CCNB1,E2F1,TP2A,TTK	
hcc5 - 2013-11-11	shared	NUPR1	transcription regulator	Inhibited	-2.333	5.55E-05	ASPM,BUB1B,CKAP2L,EXO1,HUURP,IGF2BP3,MCM10,MKI67,TRIM16	
hcc4 - 2013-11-11	shared	NUPR1	transcription regulator	Inhibited	-2.887	1.37E-02	ASPM,E2F8,FAM111B,IGF2BP3,ITPR3,KIF23,KIFC1,MKI67,MMS22L,MT1X,RIBC2,WD	
hcc4 - 2013-11-11	shared	PPARGC1A	transcription regulator	Inhibited	-2	9.30E-03	ACADL,CYP1A1,CYP1A2,CYP2A6,CYP8B1,MAOB	
hcc4 - 2013-11-11	shared	Rb	group	Inhibited	-2.39	1.52E-05	CCNE2,CDC6,CDKN2A,CYP1A1,E2F1,MYBL2,TP2A	CDKN1A,E2F1,E2F2,E2F3,E2F4,MYBL2,RB1,RBL1,Rb,TFDP1,TP53
hcc4 - 2013-11-11	shared	STAT5A	transcription regulator	Inhibited	-2.236	4.15E-03	GYS2,ID1,IGF1,IGFALS,IGHA1,SLC10A1	ESR1,NCOA3,RELA,SP1,STAT5A,TP53
hcc5 - 2013-11-11	shared	TP53	transcription regulator	Inhibited	-3.608	1.17E-12	ANLN,ASPM,BUB1B,CCNB1,CDC20,CDH1,CDKN2A,CDKN3,CENPF,E2F1,ESR1,EXO1,	E2F1,E2F4,EP300,ERBB2,FOXO1,HDAC1,IL6,MYC,RB1,TP53

									FOXP3,HJURP,MKI67,NEK2, PEG10,RECQL4,ROBO1,RR M2,TOP2A,TTK,UBE2C,UBE 2T	
hcc4 - 2013- 11-11	shared	TP53	transcription regulator	Inhibited	-2.331	1.28E-05			ABC4,ANLN,ASF1B,ASPM, C9,CCNE2,CDC6,CDKN2A,C YP1A1,DDR1,DTX1,E2F1,E2 F8,ID1,IGF1,IGF2,IGFBP2,IR F7,KIF23,MCM2,MELK,MKI 67,MT1H,MT1L,MYBL2,OAT ,ROBO1,TOP2A,TP73,UBE2C ,UBE2T,UHRF1	CDKN1A,E2F1,E2F2,E2F3,E2 F4,E2f,RB1,RBL1,Rb,TFDP1, TP53

[087] Wnt/ β -catenin signaling was the top pathway affected in both the P_{TS} and R_{TS} across all patients (Figure 2 (raw data not shown) though few genes harboring somatic mutations were shared between patients. For example, deleterious somatic genetic or genomic alterations shared between P_{TS} and R_{TS} included: HCC1: nonsilent mutations in *TP53* and *AKT2*, activation of upstream gene signatures in *CTNB1*, *E2F1* and *TCF4*, and suppression of *CDKN2A* upstream gene signature; HCC4: *CDH11* and *TGFBR3* stop codon mutations, DNA amplification in *POU5F1* and *UBD*, and DNA deletion in *GNAO1*, with activation of *E2F1* and *TXB2* upstream gene signatures and suppression of upstream gene signatures *CDKN2A* and *RBI*; HCC5: DNA deletion in *CDH1* and a nonsilent mutation in *TP53* with activation of *E2F1* and *MYC* upstream gene signatures and suppression of upstream gene signatures *CDKN2A* and *TP53*; HCC11: activating mutation S45Y in *CTNNB1*, a stop codon mutation in *LRPI*, and a nonsilent mutation in the ANK2 domain of *CDKN2A*, with DNA amplification in *SOX9*. All four patients converged on shared inactivation in P_{TS} and R_{TS} of the tumor suppressor *CDKN2A* (*p14ARF*). Using the same strategy to identify the most enriched pathways unique to the R_{TS} in all four patients, cell cycle signaling was identified (data not shown).

Example 5. Patient identity QC

[088] To verify the identity of the P_T, P_B, and P_{NAT}, as well as the R_T and R_{NAT}, a selection of 300 heterozygous single nucleotide polymorphisms (SNPs) with MAFs >0.3 and <0.7 were selected from the 1000 genomes database. Then all five DNA samples (four samples for RNA) per patient were clustered to observe any major discrepancies in subject or specimen labeling (Figure 4). Additionally, the germline variants called for both the primary and recurrent tumor samples were clustered in heatmap to verify the appropriate blocking of variant calls within a quintet of DNA specimens for each of the 4 patients (Figures 9 and 10).

Example 6. Clonal relationship (CR) value

[089] The clonal relationship of each primary and recurrent tumor pair was assessed by enumerating all nonsilent SNVs with a PolyPhen designation of medium or high for each tumor independently. The number of matched locus variants that were in-common between the two matched subject tumors was determined and the ratio of shared variants relative to the total number of variants identified for each tumor type was calculated. Subjects with CR values greater than 0.5 were considered to be of recipient origin (Figure 11).

Example 7. Donor presence within the recurrent tumor

[090] There is significant clinical importance in HCC to develop a consistent biomarker that predicts tumor recurrence and/or survival. WES and RNASeq data from the four patients as well as the P_{TS} from a larger population of HCC patients that showed tumor recurrence (n=9 inclusive of the 4; 7/9 HBV⁺) or did not (n=12; 12/12 HBV⁺) were used. Genes within regions of DNA deletions in matched P_{TS} and R_{TS} and significantly under-expressed in the P_{TS} of HCC patients who recurred (p<0.001; fold<-2; data not shown), were identified. This resulted in four genes, one of which was used in time-to-event analyses: *HERC5*.

[091] Under the assumption that the incidence of donor-transmitted malignancy is extremely low (<0.05%), the cancer clonal composition of the donor tumor biopsy should consist largely of the primary tumor (*Kauffman et al.*, Transplantation 73:358-362 (2002)). However, it is difficult to obtain pure recipient tumoral biopsy from the donor tissue using either radiofrequency ablation (HCC1, HCC4, and HCC5) or video-assisted thorascopic surgery (HCC11) techniques, as was conducted in these patients. To identify the proportion of primary tumor (recipient-based) within the donor liver biopsy, two independent calculations were conducted. The first calculation involved regressing the Vfs of all somatic (nonsilent and silent) variants called in the recurrent tumor versus those in the primary tumor. The somatic variants

unique to either the primary tumor or recurrent tumor were removed to not bias the best fit line. The slope of this curve was then calculated with least squares and subtracted from 1 to indicate a 'donor tissue contamination' proportion in the primary tumor (Figure 12).

[092] The second approach utilized a set of recipient-unique homozygous SNPs and the contamination of donor cells was calculated as the average ($1 - V_f$ s of the recipient-unique homozygous alleles in the recurrent tumor), where the alleles affected by CNVs or located in chr X/Y were excluded. The recipient-unique homozygous alleles are those alleles with $>90\%$ V_f in the recipient primary blood and $<10\%$ V_f in the donor, specific for each patient (Figure 13).

Example 8. Identification of prognostic marker

[093] Using RNASeq data, DESeq-normalized $\log_2$ gene counts in the primary tumors were scaled by the matched normal adjacent tissue within each patient. Then these fold change values for each patient were used in a contrast between patients that experience tumor recurrence ($n=9$) and patients that did not ($n=12$). A total of 273 genes were identified with $|\text{fold change}|>2$ and $p<0.01$. Then those genes with shared CNV deletions within the primary and recurrent tumor specimens in 3/4 patients in chromosome 4q (542 genes) were intersected with the 273 differentially expression genes. From this analysis, the following genes were identified: *NAA11*, *HERC5*, *DDX60*, and *HERC6*.

Example 9. Time-to-event analyses

A) Methods

[094] Time-to-event analyses were used to correlate the expression of *HERC5* with overall survival and HCC recurrence in two publically available microarray datasets (*Roessler et al.*, Cancer Res 70(24):10202-12 (2010) [GSE14520]; *Boyault et al.*, Hepatology 45(1):42-52 (2007) [E-TABM-36]). Three different analyses were conducted for each study: a Kaplan-Meier (KM) analysis of the difference between two survival curves using a chi-squared test of equality,

a multivariate Cox proportion hazard (PH) regression model, and a univariate Cox PH regression model for each variable. For the Roessler study, *HERC5* (219863_at on HGU133A array) was divided into high or low patient groups using mean-2SD of the healthy normal distribution (n=239) as the cut point. There were 62 HCC patients in the low group and 180 patients in the high group. The difference between high and low expression groups for each gene was assessed using the p-value for the grouping coefficient, the hazard ratio, as well as the likelihood ratio test, after adjusting for age, cirrhosis status (binary), gender (binary), HBV/HCV status (active viral replication chronic carrier=2; chronic carrier=1; no=0), and TNM staging (I=1, II=2, IIIA/IIIB/IIIC=3) for the multivariate Cox PH regression model. The same variables were also assessed individually in univariate Cox PH regression models. Both overall survival and time to tumor recurrence were assessed in two separate analyses (Figure 3 & Tables 6A-C). For the Boyault study, there were no normal healthy subject samples, so *HERC5* (219863_at on HGU133A array) was cut into high and low groups using the median of the HCC patient expression values. There were 20 HCC patients in the low group and 24 patients in the high group. Then both overall survival and progression-free survival between the high and low patient groups was computed adjusting for gender (binary), age, and HBV (titer negative=0; titer positive=1) status (Figure 3 & Tables 7A-C).

Table 6A: Multivariate Cox PH model regression table predicting survival or HCC recurrence for Roessler et al., HGU133A HCC study using HERC5 high/low groupings													
Survival	coef	Exp (coef)	Se (coef)	z	Pr(> z)	lower .95	upper .95	Likelihood ratio test	Wald test	Logrank test	total (n)	events (n)	missing (n)
HERC5 (Low)	0.7044	2.0226	0.2419	2.9123	0.0036	1.2590	3.2492	p=2.732e-09	p=2.052 e-08	p=1.583 e-09	224	86	23
Gender (M, F)	0.3074	1.3598	0.3809	0.8068	0.4198	0.6445	2.8691						
Cirrhosis (No, Yes)	-1.2788	0.2784	0.7213	1.7728	0.0763	0.0677	1.1445						
Age	-0.0002	0.9998	0.0107	0.0144	0.9885	0.9790	1.0211						
HBV (AVR-CC, CC, No)	0.2584	1.2949	0.2262	1.1426	0.2532	0.8312	2.0173						
TNM Staging (I, II, III)	0.8433	2.3241	0.1474	5.7202	0.00000011	1.7409	3.1028						
HCC recurrence	coef	Exp (coef)	Se (coef)	z	Pr(> z)	lower .95	upper .95	Likelihood ratio test	Wald test	Logrank test	total (n)	events (n)	missing (n)
HERC5 (Low)	0.5881	1.8005	0.2052	2.8655	0.0042	1.2042	2.6921	p=3.98e-07	p=1.01 e-06	p=4.55 e-07	224	125	23
Gender (M, F)	0.7270	2.0688	0.3348	2.1717	0.0299	1.0734	3.9872						
Cirrhosis (No, Yes)	-0.5756	0.5624	0.4231	1.3603	0.1737	0.2454	1.2888						
Age	0.0060	1.0060	0.0088	0.6881	0.4914	0.9889	1.0234						
HBV (AVR-CC, CC, No)	0.3062	1.3583	0.1914	1.5996	0.1097	0.9333	1.9768						
TNM Staging (I, II, III)	0.5548	1.7416	0.1172	4.7331	0.00000022	1.3841	2.1914						

Table 6B: Kaplan-Meier table predicting survival or HCC recurrence for Roessler et al., HGU133A HCC study using HERC5 high/low groupings							
Survival	N	Obs	Exp	(O-E)^2/E	(O-E)^2/N	median	P
HERC5 (High)	180	64	75	1.63	7.47	NA	0.0063
HERC5 (Low)	62	32	21	5.83	7.47	42.2	
HCC recurrence	N	Obs	Exp	(O-E)^2/E	(O-E)^2/N	median	P
HERC5 (High)	180	96	107.1	1.15	5.43	49.1	0.0198
HERC5 (Low)	62	40	28.9	4.26	5.43	23	

Table 6C: Univariate Cox PH regression model table predicting survival or HCC recurrence for Roessler et al., HGU133A HCC study using HERC5 high/low groupings						
Variable	Survival			Recurrence		
	HR (95% CI)	P		HR (95% CI)	P	
HBV (AVR-CC, CC, No)	1.318 (0.855,2.032)	0.212		1.236 (0.855,1.787)	0.259	
TNM Staging (I, II, III)	2.340 (1.771,3.091)	2.18E-09		1.758 (1.405,2.198)	7.81E-07	
Age (<50)	1.258 (0.842,1.878)	0.262		1.008 (0.719,1.415)	0.963	
Gender (M, F)	1.859 (0.0901,3.834)	0.0933		2.361 (1.239,4.496)	0.009	
Cirrhosis (No, Yes)	0.196 (0.048,0.797)	0.0227		0.499 (0.233,1.068)	0.073	
HERC5 (Low, High)	1.793 (1.173,2.743)	0.00706		1.545 (1.068,2.235)	0.0208	

Table 7A Multivariate Cox PH model regression table predicting overall survival and PFS for Boyault et al., HGU133A HCC study using HERC5 high/low groupings													
Survival	coef	Exp (coef)	Se (coef)	z	Pr(> z)	lower .95	upper .95	Likelihood ratio test	Wald test	Logrank test	total (n)	event s (n)	missing (n)
HERC5 (Low)	1.1971	3.3104	0.5079	2.3570	0.0184	1.2234	8.9578	0.198	0.2034	0.1644	41	20	24
HBV (Yes, No)	0.2506	1.2849	0.5167	0.4851	0.6276	0.4667	3.5371						
Gender (M, F)	0.1949	1.2152	0.5931	0.3286	0.7425	0.3800	3.8860						
Age	0.0161	1.0162	0.0200	0.8069	0.4197	0.9772	1.0568						
PFS	coef	Exp (coef)	Se (coef)	z	Pr(> z)	lower .95	upper .95	Likelihood ratio test	Wald test	Logrank test	total (n)	event s (n)	missing (n)
HERC5 (Low)	1.3348	3.7991	0.5151	2.5910	0.0096	1.3842	10.4273	0.1264	0.1313	0.0971	41	20	24
HBV (Yes, No)	0.1517	1.1639	0.5360	0.2831	0.7771	0.4071	3.3274						
Gender (M, F)	0.2785	1.3211	0.6026	0.4621	0.6440	0.4055	4.3037						
Age	0.0175	1.0177	0.0209	0.8380	0.4020	0.9768	1.0603						

Table 7B. Kaplan-Meier table predicting overall survival or PFS for Boyault et al., HGU133A HCC study using HERC5 high/low groupings									
Survival	N	Obs	Exp	(O-E)^2/E	(O-E)^2/V	events	median	P	
HERC5 (High)	24	8	13	1.93	5.16	8	NA	0.0231	
HERC5 (Low)	20	13	8	3.13	5.16	13	24		
PFS	N	Obs	Exp	(O-E)^2/E	(O-E)^2/V	events	median	P	
HERC5 (High)	24	15	19.6	1.09	3.35	15	36	0.0674	
HERC5 (Low)	20	15	10.4	2.06	3.35	15	13		

Table 7C. Univariate Cox PH regression model table predicting overall survival or PFS for Boyault et al., HGU133A HCC study using HERC5 high/low groupings				
	Survival		PFS	
Variable	HR (95% CI)	P	HR (95% CI)	P
HBV (Yes, No)	1.315 (0.509,3.395)	0.572	1.019 (0.437,2.383)	0.964
Gender (M, F)	1.008 (0.336,3.029)	9.88E-01	1.295 (0.491,3.411)	6.01E-01
Age (<65)	1.091 (0.446,2.671)	0.848	1.483 (0.695,3.166)	0.309
<i>HERC5</i> (Low, High)	2.686 (1.108,6.508)	0.0287	1.962 (0.951,4.045)	0.068

[095] To demonstrate prognostic specificity of *HERC5* compared to other genes within the same DNA deletion region in chromosome 4q, a similar time-to-event analysis was conducted on each of the genes found within this deleted region from one of the independent studies (*Roessler et al.*, Cancer Res 70(24):10202-12 (2010)). Of the 262 genes identified on 4q and represented on the array, 118 were under-expressed in the tumor biopsies compared to tissue from the normal controls. Using these 118 genes in independent time-to-event models, *HAND2* had the most significant association with HCC recurrence, although it had a highly unbalanced distribution of high/low patients (3.6% of patients in the low group). *HERC5* was the second most significant correlate with HCC recurrence (Figure 7), demonstrating biological specificity of this gene independent of other genes with CN deletions in chromosome 4q.

B) Results

[096] The top ranked upstream gene signatures among all suppressed genes included IFN α 2 and IFN- λ . *HERC5* mRNA expression was categorized in time-to-event analysis from *Roessler et al.* and *Boyault et al.*, where *HERC5* mRNA expression was categorized into high (n=180) or low (n=62) HCC patient groups using healthy control liver tissue (n=239)(*Roessler*) or the median (low n=20; high n=24) of the HCC patient distribution (*Boyault*). After adjustment for age, cirrhosis status, gender, HBV/HCV status and TNM staging, the high/low grouping variable had p=0.004; HR=2.02 [1.26,3.25] and p=0.004; HR=1.80 [1.20,2.69] for predicting overall survival and HCC recurrence, respectively in the *Roessler* study (Figure 3 & Tables 6A-

C) and adjusting for age, gender, and HBV status, the high/low grouping variable had $p=0.02$; $HR=3.31$ [1.22, 8.96] and $p=0.01$; $HR=3.80$ [1.38, 10.43] for predicting overall survival and progression-free survival, respectively in the Boyault study (Figure 3 and Tables 7A-C).

Specificity of *HERC5* prognostic correlation compared to other genes in the deleted region of chromosome 4q was assessed (Figure 7). *HERC5* was also significantly over-expressed ($p<0.0001$) in the molecularly-determined good prognosis ($n=109$) compared to poor prognosis group ($n=107$) identified by *Hoshida et al.*, *Gastroenterology* 144:1024-30 (2013), in patients with HCV-positive cirrhosis (Figure 8).

[097] Results indicate a clonal advantage in R_{TS} post-OLT compared to P_{TS} in HCC, with Wnt/ β -catenin signaling most activated in both tumors. *HERC5* was identified as a predictor of risk of HCC recurrence and survival and is not present in any of the 14 gene signatures catalogued in the Liverome database (*Lee et al.*, *BMC Genomics* 12 Suppl 3:S3 (2011)). The protein coded by this gene catalyzes the conjugation of ISG15 and drives sustained activation of IRF3. Thus host anti-viral responses are activated by the presence of *HERC5* and reduced by its absence, suggesting an association with regulation of innate immune responses, a potential critical function leading to tumor recurrence in HCC; this hypothesis will have to be rigorously evaluated in future studies.

Example 10. DNA and RNA Hepatitis B viral integration

[098] For DNA integration sites of the hepatitis B virus (HBV) into the human genome, the 90mer WES paired end sequence reads were mapped to a reference index file including the human genome (UCSC hg19; Feb 2009 release; Genome Reference Consortium GRCh37), hepatitis A virus (NC_001489), hepatitis B virus (NC_003977), hepatitis C virus (NC_004102), hepatitis delta virus (NC_001653), hepatitis E virus (NC_001434), and hepatitis G virus (NC_001710) using Bowtie2 (v2.0.0-beta7; *Langmead et al.*, *Nat Methods* 9(4):357-9

(2012)). Read pairs containing one mate aligning to the human genome and another mate aligning to the HBV genome were retained, as were instances where both read pairs aligned to the HBV genome. At least 2 events had to be observed for each approach to be considered significant, consistent with a previous report (*Sung et al.*, Nat Genet 27(44):765-9 (2012)). For RNA reads, a similar methodology was conducted, using 90mer paired end reads and TopHat2 as the read aligner (v2.0.9; *Kim et al.*, Genome Biol 14(4):R36 (2013)). Results are represented in Tables 8A-B.

Table 8A: WES and RNA-Seq counts of aligned mates within the HBV genome	
Sample	WES HBV counts (RNASeq counts)
HCC1-D-13T-1	6 (34)
HCC1-D-13T-12	0 (14)
HCC4-D-13T-4	11 (14450)
HCC4-D-13T-15	12 (16000)
HCC5-D-13T-5	0 (8)
HCC5-D-13T-16	0 (0)
HCC11-D-13T-11	0 (1)
HCC11-D-13T-22	0 (0)

Table 8B: WES and RNA-Seq HBV integration events observed							
Data	Chromosome	Region Start	HBV Region Start	Sample	Human Gene	HBV Gene	Number of Events Within Region
WES	chr8	42202427	1790	HCC1-D-13T-1	POLB	NA	2
WES	chr8	43093811	2639	HCC1-D-13T-1	NA	NA	2
WES	chr10	128813944	1043	HCC1-D-13T-1	DOCK1	NA	2
RNASeq	chr17	19499171	1581	HCC1-R-13T-1	NA	NA	2
RNASeq	chr8	36915003	2379	HCC4-R-13T-4	NA	Polymerase	2
RNASeq	chr8	112045043	1574	HCC4-R-13T-4	NA	Polymerase, X gene	179
RNASeq	chr19	13054759	599	HCC4-R-13T-4	CALR // RAD23A	Polymerase, large S protein, middle S protein	2
RNASeq	chr8	112045043	1527	HCC4-R-13T-15	NA	Polymerase, X gene	242

[0099] WES and RNASeq data were used to identify both sites of human genome integration and reads with both mates mapping to virus using similar methods that have been reported previously (*Sung et al.*, Nat Genet 27(44):765-9 (2012); *Jiang et al.*, Genome Res 22:593-601 (2012)). For HBV presence, HCC4 primary and recurrent tumors had the most DNA and RNA HBV counts (11/14,450 and 12/16,000, for primary and recurrent tumor DNA/RNA, respectively), which was consistent with the clinical assessment. HCC1 had only DNA HBV counts in the P_T and not R_T, consistent with a negative viral titer, though RNA HBV counts were observed in this R_T (14 counts; Table 8A). Both HCC5 and HCC11 had no DNA HBV counts in P_T or R_T, consistent with the clinical assessment. HBV integration sites were observed in 3 unique regions in HCC1 P_T DNA and one with RNA. For HCC4, 3 unique sites in the P_T RNA were observed with the most integration counts between HBV polymerase/X gene and chromosome 8q23.2. This same hyper-integrated site was also observed in HCC4 R_T RNA. (Table 8B).

Example 11. Determination of HERC5 Level in a HCC Sample

[0100] A first patient is diagnosed with hepatocellular carcinoma and decisions are being made regarding this patient's treatment options. The patient is evaluated for traditional clinical criteria including tumor size, number of tumors, major vascular involvement, and whether there are any extrahepatic lesions. The patient is found to have no major vascular involvement, no extrahepatic lesions and two hepatic lesions with a diameter of 2 cm each.

[0101] A needle biopsy of one of the lesions is made, the lesion chosen is the one most easily accessed during the needle biopsy. The HERC5 mRNA expression level is determined using qPCR and a labeled probe specific for HERC5. The hepatocellular carcinoma HERC5 mRNA expression level is compared to a standardized cutoff value.

[0102] The cutoff value is determined by determining HERC5 mRNA expression level in a pool of normal patients using the same qPCR technique. The cutoff value is determined to be two standard deviations below the mean of the normal patient control group values.

[0103] The first patient has a HERC5 level that is lower than the cutoff value and is deprioritized on the transplantation list.

[0104] A second patient is diagnosed with hepatocellular carcinoma and evaluation progresses similarly to the first patient. The patient is found to have no major vascular involvement, no extrahepatic lesions and three hepatic lesions with a diameter of 1 cm, 1.5 cm, and 2 cm, respectively. The second patient has a HERC5 level that is higher than the cutoff value and is prioritized on the transplantation list.

EQUIVALENTS

[0105] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiments may be practiced in many ways and the claims include any equivalents thereof.

WHAT IS CLAIMED IS:

1. A method of treating hepatocellular carcinoma in a patient comprising:
 - a. obtaining a hepatocellular carcinoma sample from the patient;
 - b. determining whether the sample has a normal level of HERC5 mRNA expression;
 - c. qualifying the patient for an orthotopic liver transplant if the sample has a normal level of HERC5 mRNA expression.
2. The method of claim 1, wherein the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a historical control group.
3. The method of claim 2, wherein the historical control group has normal subjects.
4. The method of claim 2, wherein the historical control group has patients who had a hepatocellular carcinoma that did not recur within two years.
5. The method of any one of claims 3-4, wherein the HERC5 patient sample level is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical control group.
6. The method of any one of claims 3-4, wherein the HERC5 patient sample is designated as normal if it is equal to or higher than one standard deviation below the mean of the historical control group.
7. The method of claim 2, wherein the historical control group has patients who had a hepatocellular carcinoma that recurred within two years.
8. The method of claim 7, wherein the HERC5 patient sample level is designated as normal if it is equal to or higher than one standard deviation above the mean of the historical control group.
9. The method of claim 2, wherein the historical control group has patients who have had hepatocellular carcinoma.
10. The method of claim 9, wherein the HERC5 patient sample is designated as normal if it is higher than the median of the historical group.
11. The method of any one of claims 2-10, wherein the historical control comprises data from at least 10 individuals.
12. The method of any one of claims 2-10, wherein the historical control comprises data from at least 25 individuals.
13. The method of any one of claims 2-10, wherein the historical control comprises data from at least 50 individuals.

14. The method of claim 1, wherein the level of HERC5mRNA in the sample is compared to the level of HERC5 mRNA in a sample of normal tissue obtained from the patient.
15. The method of claim 14, wherein the normal tissue is liver tissue.
16. The method of any one of claims 14-15, wherein the level of HERC5 mRNA in the hepatocellular carcinoma sample is higher than 80% of the level in the normal tissue sample and the patient qualifies for an orthotopic liver transplant.
17. The method of any one of claims 14-15, wherein the level of HERC5 mRNA in the hepatocellular carcinoma sample is lower than 50% of the level in the normal tissue sample and the patient is disqualified from an orthotopic liver transplant.
18. The method of any one of claims 1-17, wherein the patient does not qualify for sorafenib.
19. The method of any one of claims 1-17, wherein the patient was treated with sorafenib and the treatment was unsuccessful.
20. The method of any one of claims 1-19, wherein the hepatocellular carcinoma sample has a HERC deletion and the patient is not qualified for an orthotopic liver transplant.
21. The method of any one of claims 1-19, wherein the hepatocellular carcinoma sample has HERC5 underexpression and the patient is not qualified for an orthotopic liver transplant.
22. The method of any one of claims 1-21, wherein the decision to qualify a patient for an orthotopic liver transplant also includes an evaluation of clinical criteria.
23. The method of claim 22, wherein the clinical criteria comprise at least one of the size of the lesion, the number of lesions, whether there are extrahepatic manifestations, whether there is vascular invasion, whether the HCC was poorly differentiated on biopsy, tumor state, tumor grade, and alpha-fetoprotein levels.
24. The method of any one of claims 22-23, wherein the clinical criteria comprise
 - a. a single lesion not exceeding 5 cm or no more than 3 lesions, none greater than 3 cm; and
 - b. no macrovascular invasion.
25. The method of any one of claims 22-23, wherein the clinical criteria comprise:
 - a. a single lesion not greater than 6.5 cm; or
 - b. no more than 3 lesions, none of which exceed 4.5 cm and whose total tumor diameter does not exceed 8 cm.
26. The method of any one of claims 22-23, wherein the clinical criteria comprise the Toronto criteria.

27. The method of any one of claims 22-23, wherein the clinical criteria comprise the Hangzhou criteria.
28. The method of any one of claims 22-27, wherein the α -fetoprotein concentration is less than 400 ng/mL.
29. The method of any one of claims 1-28, wherein a patient with a normal level of HERC5 mRNA is given priority on a transplant waiting list over any hepatocellular carcinoma patient without a normal level of HERC5 mRNA.
30. The method of any one of claims 1-16, 18-19, 22-29, wherein the patient receives a liver transplant.
31. A method of evaluating the recurrence risk profile of a hepatocellular carcinoma comprising:
 - a. providing a hepatocellular carcinoma sample;
 - b. determining *in vitro* whether the sample has a normal level of HERC5 mRNA expression;
 - c. qualifying the hepatocellular carcinoma as having a low risk of recurrence if the sample has a normal level of HERC5 mRNA expression and the determination is made in conjunction with at least one clinical criteria.
32. The method of claim 31, wherein the hepatocellular carcinoma has a low risk of recurrence after an orthotopic liver transplant.
33. The method of claim 31-32, wherein the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a historical control group.
34. The method of claim 33, wherein the historical control group has normal subjects.
35. The method of claim 33, wherein the historical control group has patients who had a hepatocellular carcinoma that did not recur within two years.
36. The method of any one of claims 34-35, wherein the HERC5 patient sample level is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical control group.
37. The method of any one of claims 35-35, wherein the HERC5 patient sample is designated as normal if it is equal to or higher than one standard deviation below the mean of the historical control group.
38. The method of claim 33, wherein the historical control group has patients who had a hepatocellular carcinoma that recurred within two years.

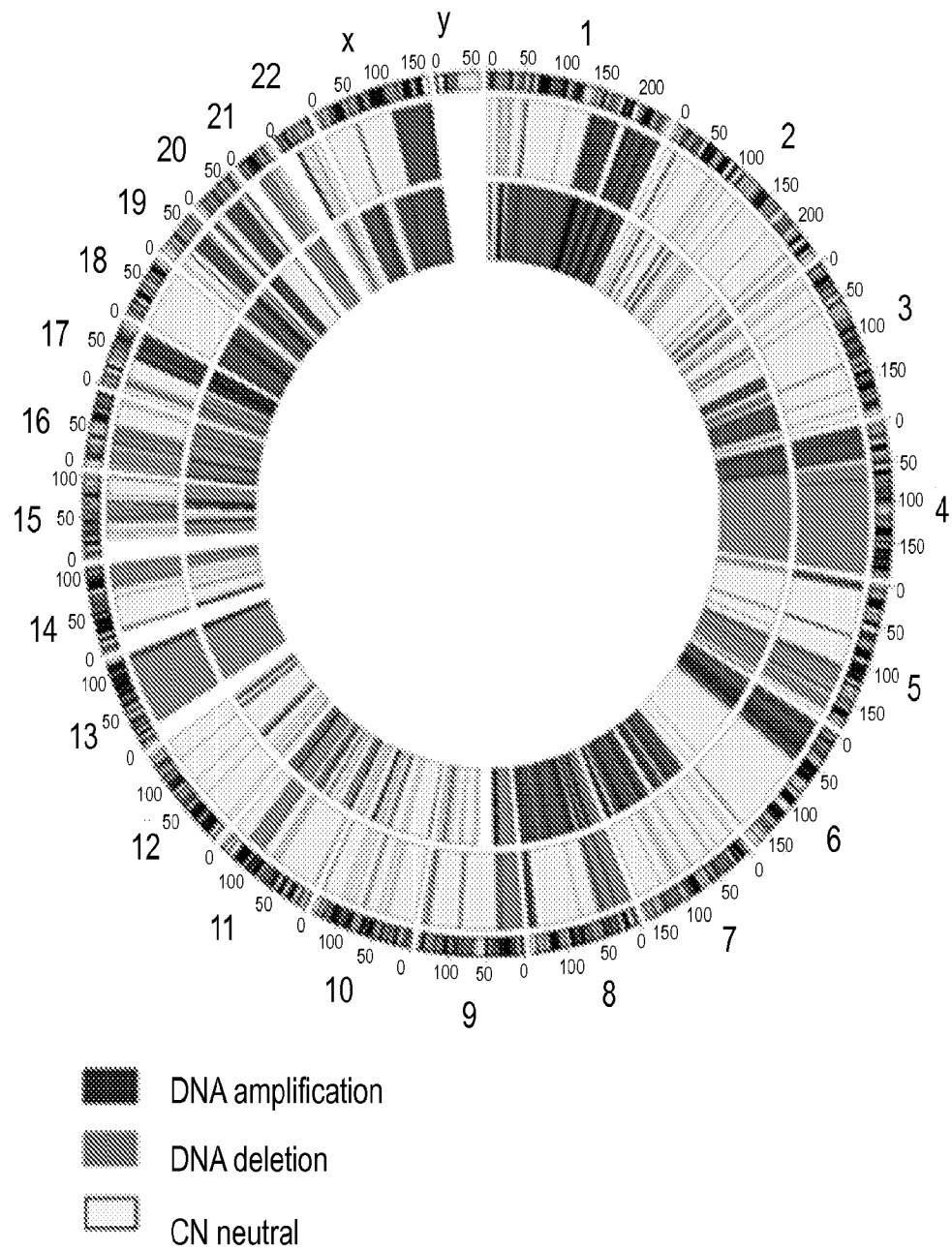
39. The method of claim 38, wherein the HERC5 patient sample level is designated as normal if it is equal to or higher than one standard deviation above the mean of the historical control group.
40. The method of claim 33, wherein the historical control group has patients who have had hepatocellular carcinoma.
41. The method of claim 40, wherein the HERC5 patient sample is designated as normal if it is higher than the median of the historical group.
42. The method of any one of claims 33-41, wherein the historical control comprises data from at least 10 individuals.
43. The method of any one of claims 33-41, wherein the historical control comprises data from at least 25 individuals.
44. The method of any one of claims 33-41, wherein the historical control comprises data from at least 50 individuals.
45. The method of claim 31, wherein the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a sample of normal tissue obtained from the patient.
46. The method of claim 45, wherein the normal tissue is liver tissue.
47. The method of any one of claims 45-46, wherein the level of HERC5 mRNA in the hepatocellular carcinoma sample higher than 80% of the level in the normal tissue sample and the patient qualifies for an orthotopic liver transplant.
48. The method of any one of claims 45-46, wherein the level of HERC5 mRNA in the hepatocellular carcinoma sample is lower than 50% of the level in the normal tissue sample and the patient is disqualified from an orthotopic liver transplant.
49. The method of any one of claims 31-48, wherein the patient does not qualify for sorafenib.
50. The method of any one of claims 31-48, wherein the patient was treated with sorafenib and the treatment was unsuccessful.
51. The method of any one of claims 31-48, wherein the hepatocellular carcinoma sample has a HERC5 deletion and has a high risk of recurrence after an orthotopic liver transplant.
52. The method of any one of claims 31-48, wherein the hepatocellular carcinoma sample has HERC5 underexpression and has a high risk of recurrence after an orthotopic liver transplant.
53. The method of any one of claims 31-52, wherein evaluation of the risk of recurrence also includes an evaluation of clinical criteria.

54. The method of claim 22, wherein the clinical criteria comprise at least one of the size of the lesion, the number of lesions, whether there are extrahepatic manifestations, whether there is vascular invasion, whether the HCC was poorly differentiated on biopsy, tumor state, tumor grade, and alpha-fetoprotein levels.
55. The method of any one of claims 22-23, wherein the clinical criteria comprise
 - a. a single lesion not exceeding 5 cm or no more than 3 lesions, none greater than 3 cm; and
 - b. no macrovascular invasion.
56. The method of any one of claims 22-23, wherein the clinical criteria comprise:
 - a. a single lesion not greater than 6.5 cm; or
 - b. no more than 3 lesions, none of which exceed 4.5 cm and whose total tumor diameter does not exceed 8 cm.
57. The method of any one of claims 22-23, wherein the clinical criteria comprise the Toronto criteria and include any tumor size or number and no systemic symptoms as a result of HCC and histological-based exclusion of poorly differentiated HCC (beyond Milan tumors only).
58. The method of any one of claims 22-23, wherein the clinical criteria comprise the Hangzhou criteria.
59. The method of any one of claims 22-27, wherein the α -fetoprotein concentration is less than 400 ng/mL.
60. The use of a HERC5 forward and reverse primer for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.
61. The use of a HERC5-specific probe for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.
62. The use of a HERC5 nucleic acid sequence as a quantitative control for producing a diagnosticum for the *in vitro* diagnosis of hepatocellular carcinoma that may be treated with an orthotopic liver transplant.
63. A method of identifying a patient as a candidate for orthotopic liver transplant comprising:
 - a. obtaining a hepatocellular carcinoma sample from the patient;
 - b. determining whether the sample has a normal level of HERC5 mRNA expression.

64. The method of claim 63, wherein the level of HERC5 mRNA in the sample is compared to the level of HERC5 mRNA in a historical control group.
65. The method of claim 64, wherein the historical control group has normal subjects.
66. The method of claim 64, wherein the historical control group has patients who had a hepatocellular carcinoma that did not recur within two years.
67. The method of any one of claims 65-66, wherein the HERC5 patient sample level is designated as normal if it is equal to or higher than two standard deviations below the mean of the historical control group.

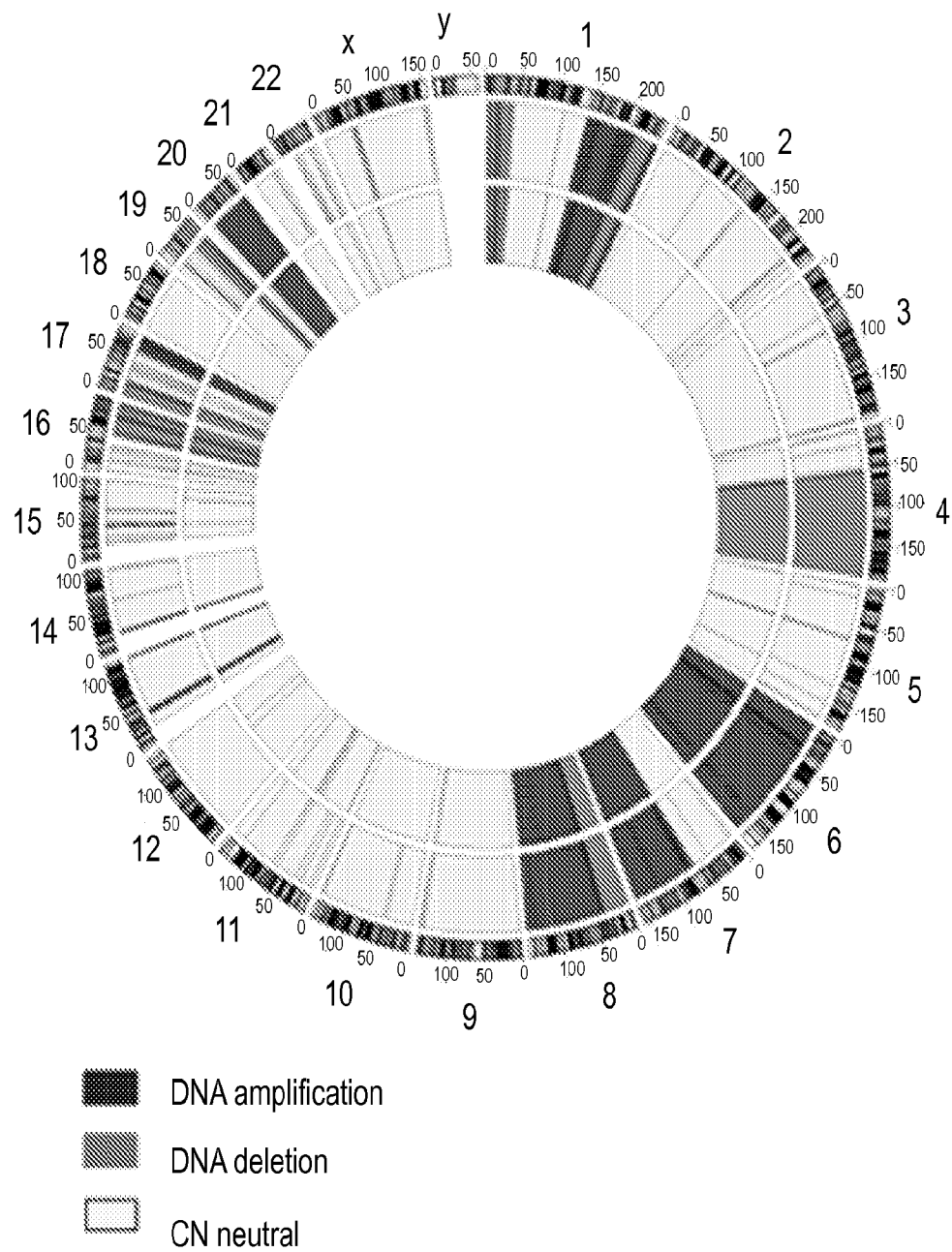
1/20

FIG. 1A



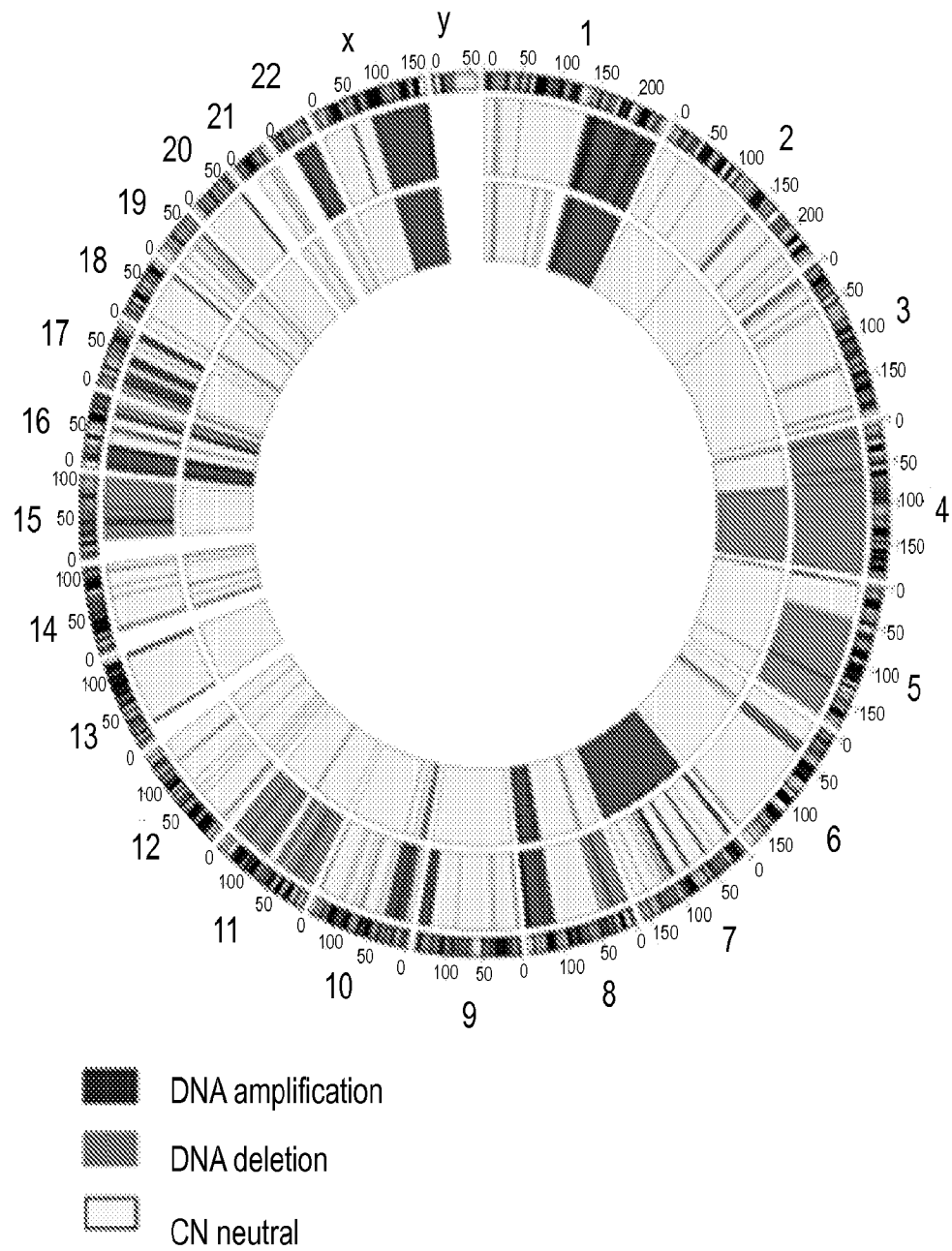
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FIG. 1B



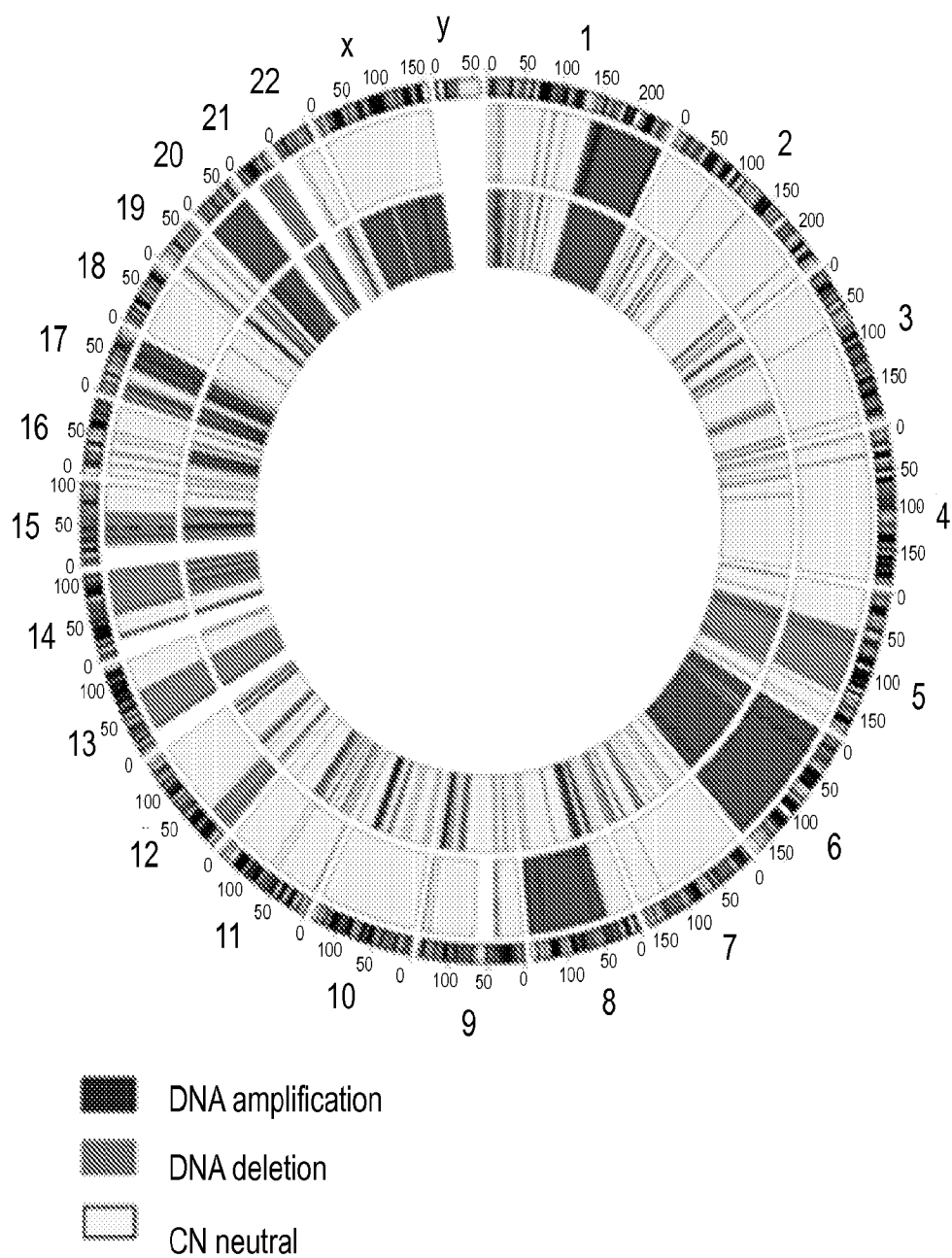
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FIG. 1C



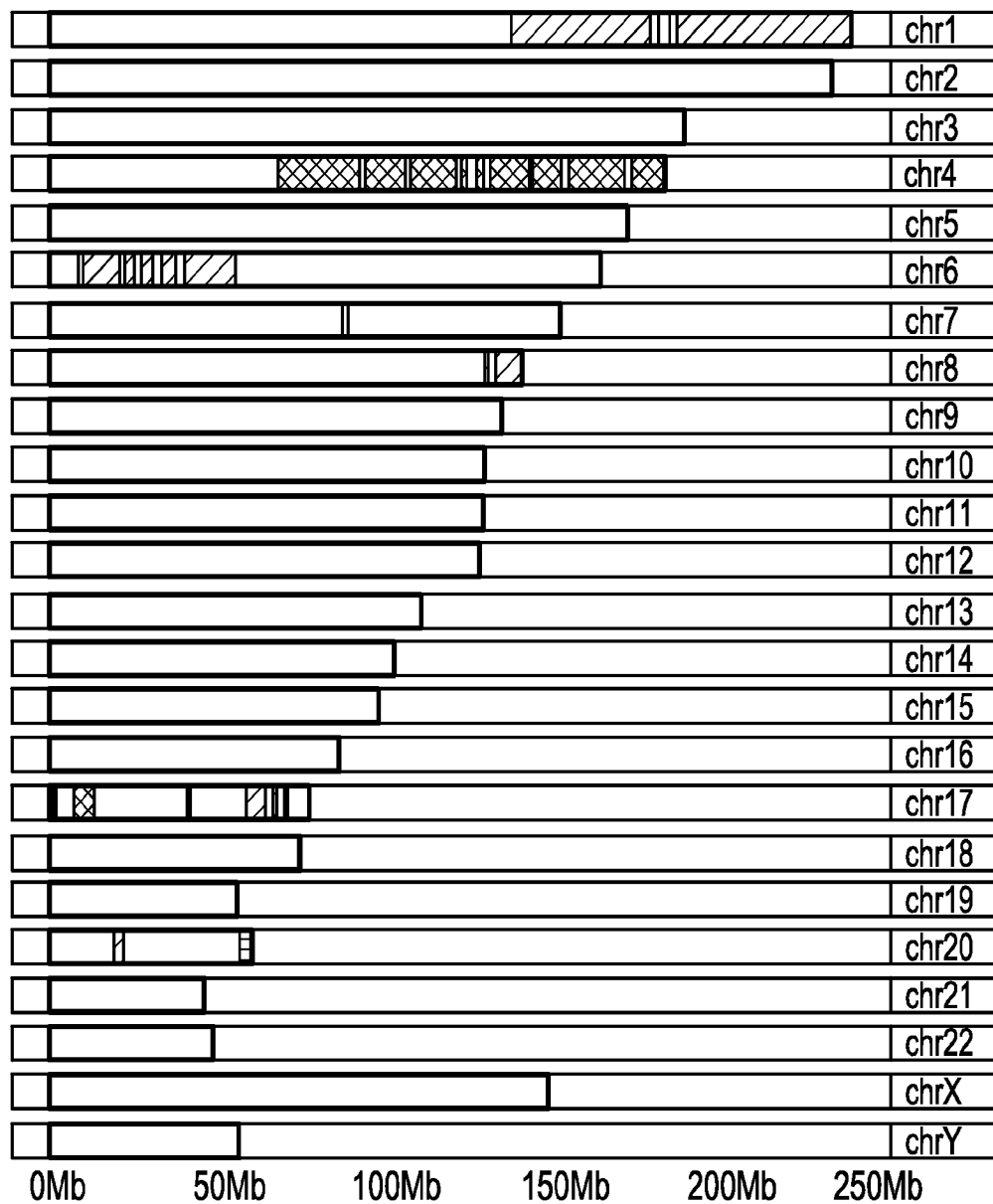
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FIG. 1D



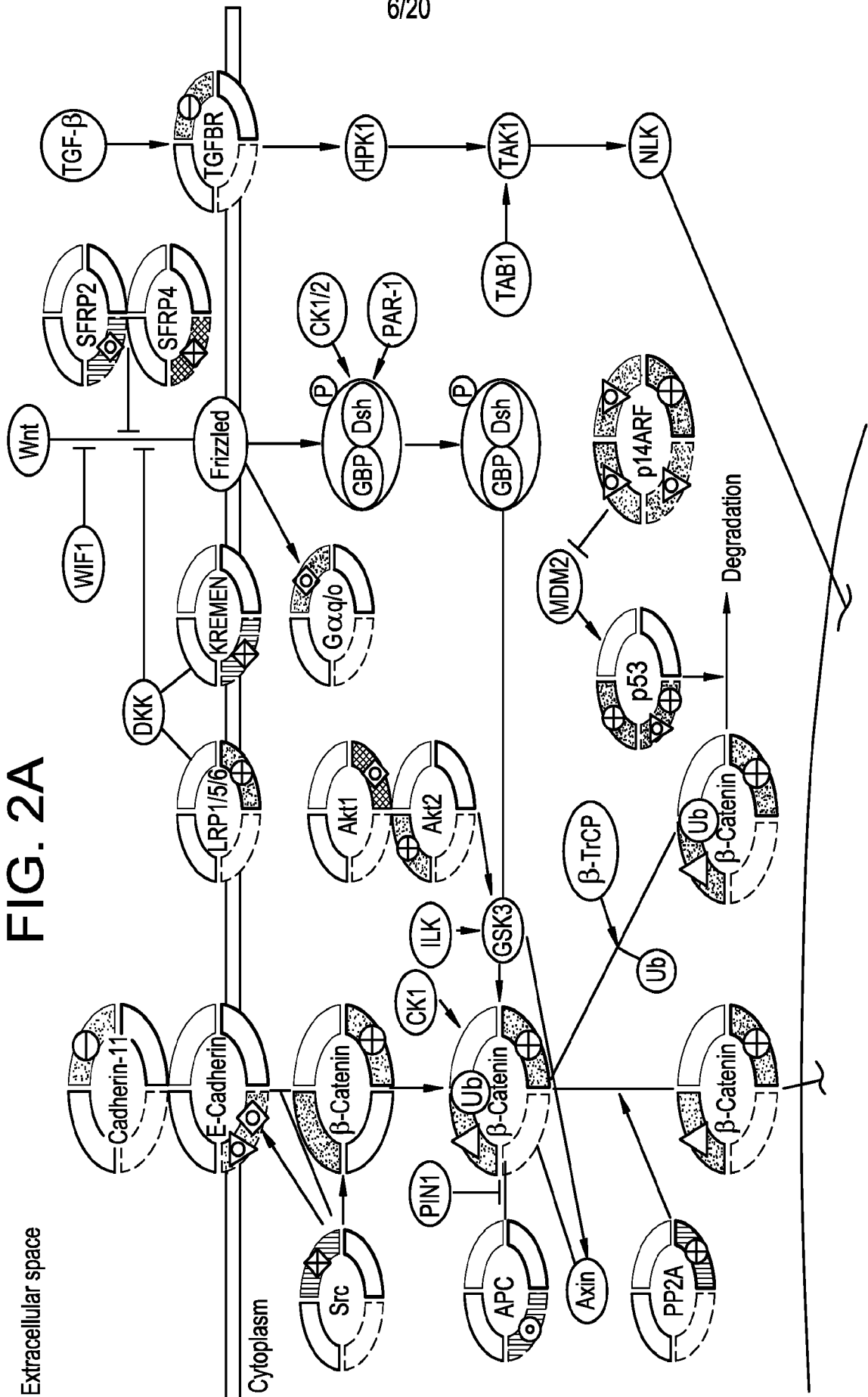
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FIG. 1E



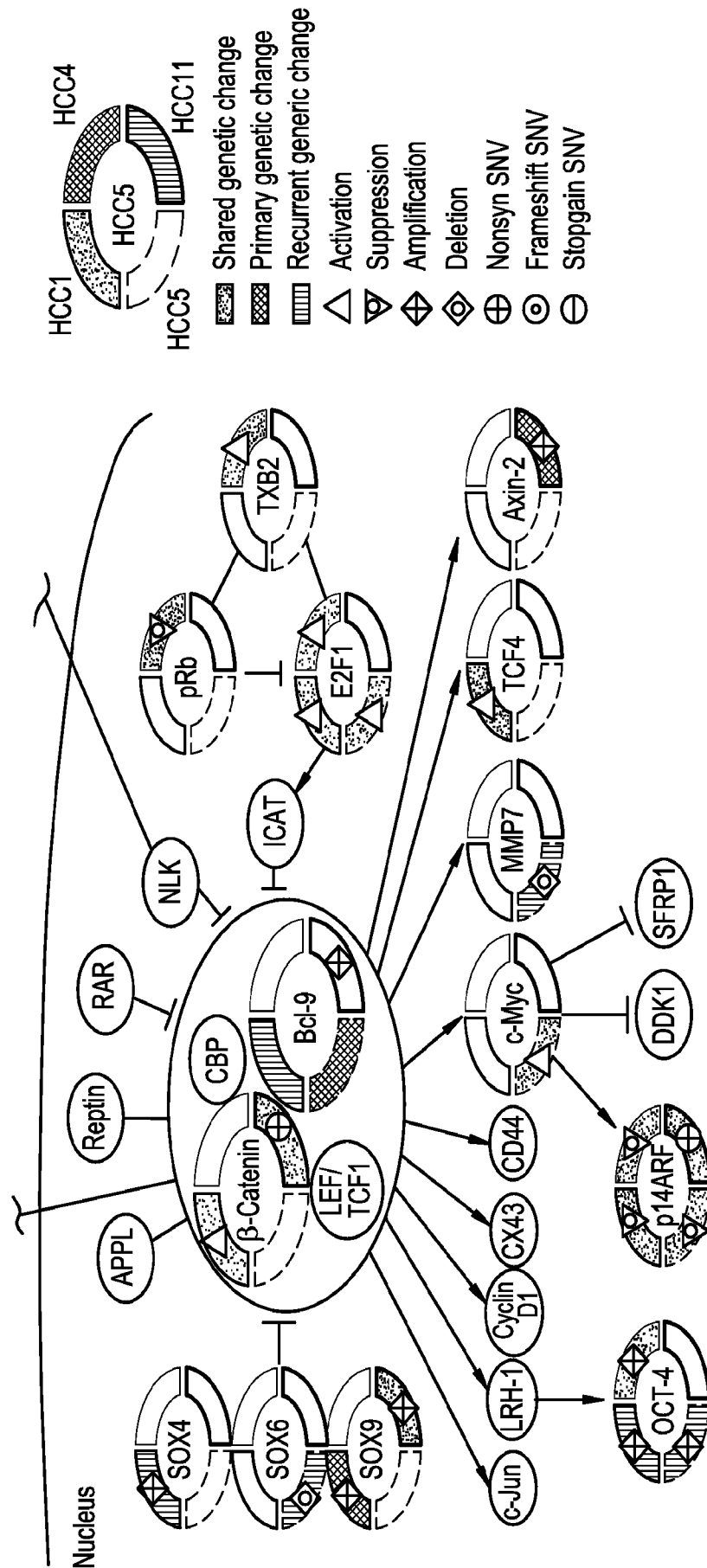
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FIG. 2A



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FIG. 2B



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FIG. 3A

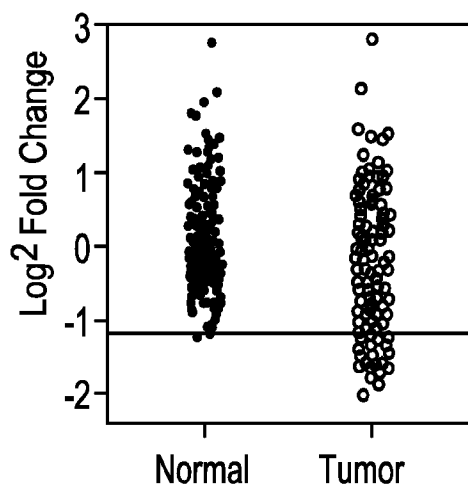


FIG. 3B

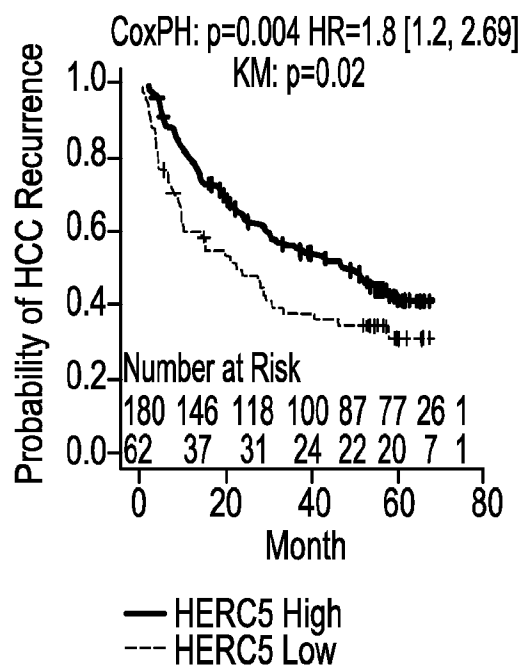


FIG. 3C

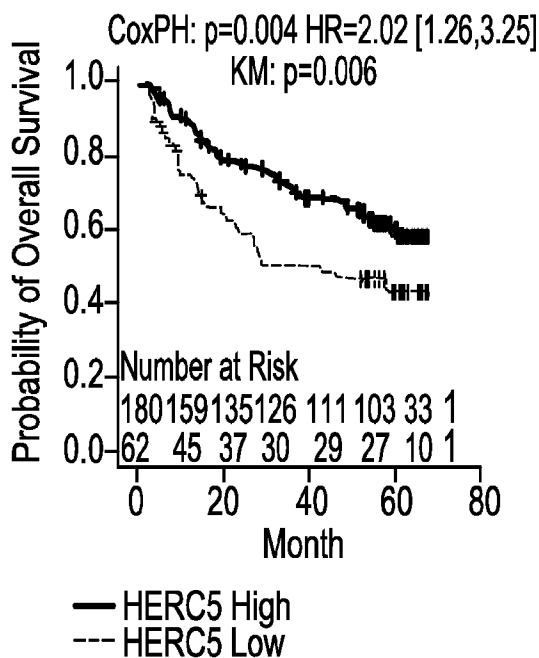
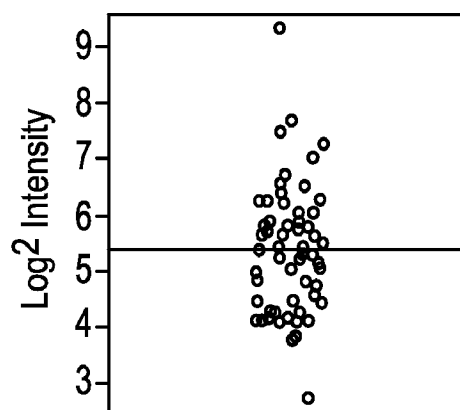


FIG. 3D



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FIG. 3E

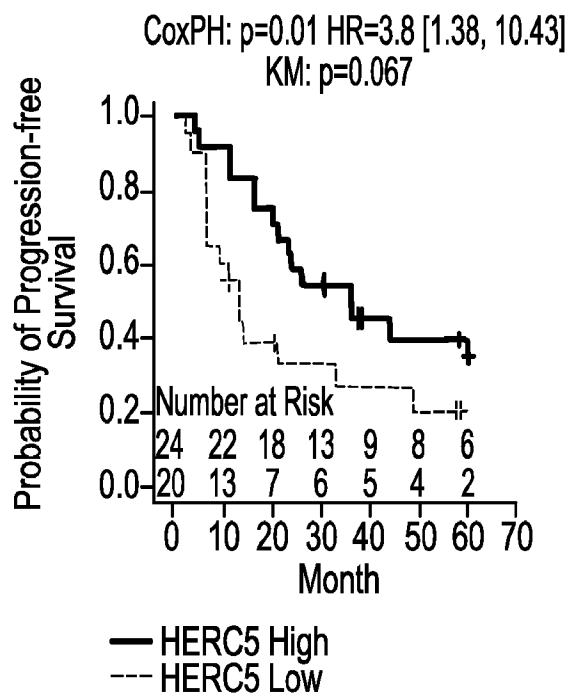
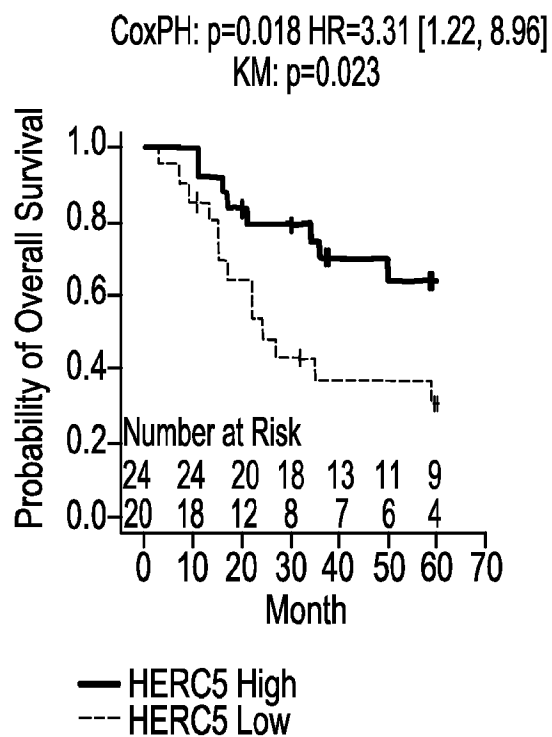
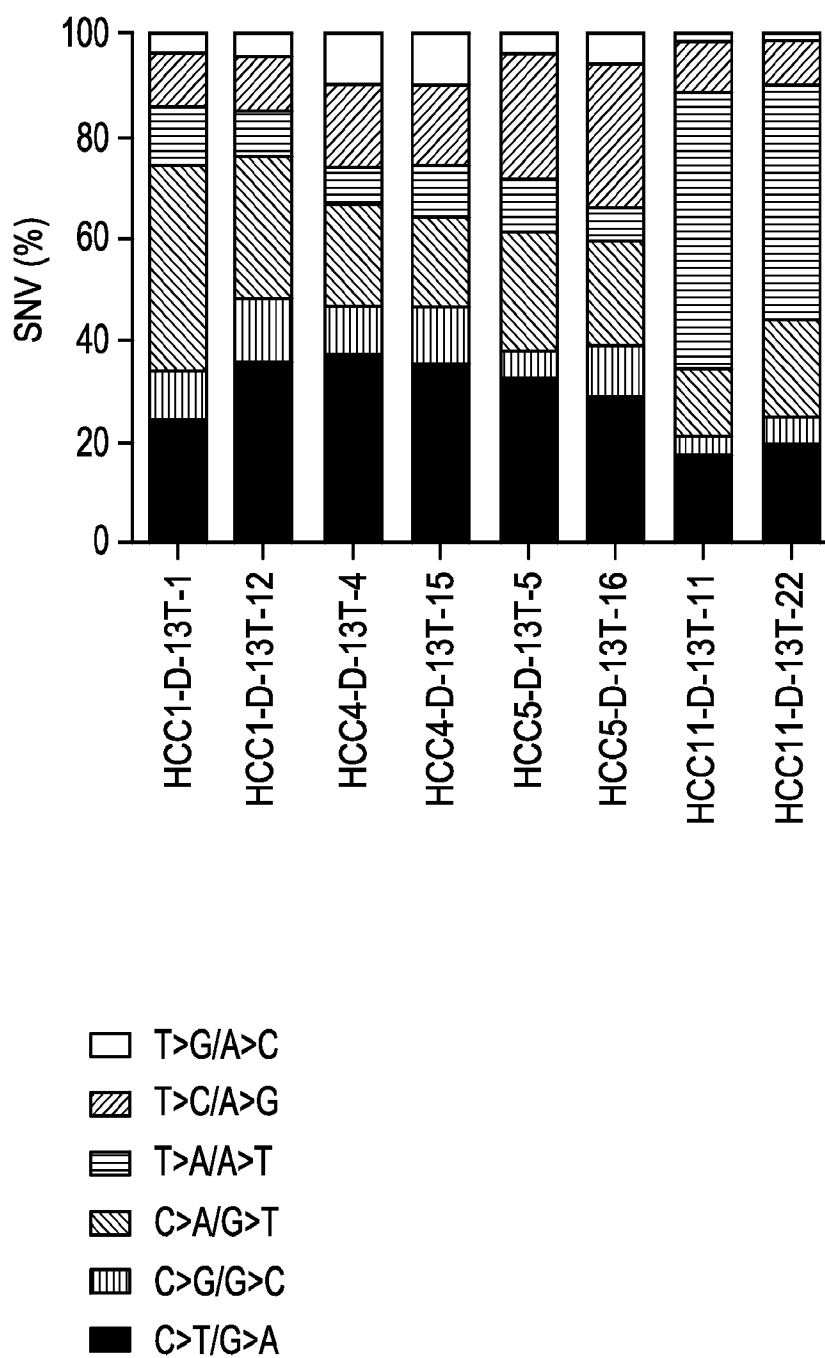


FIG. 3F



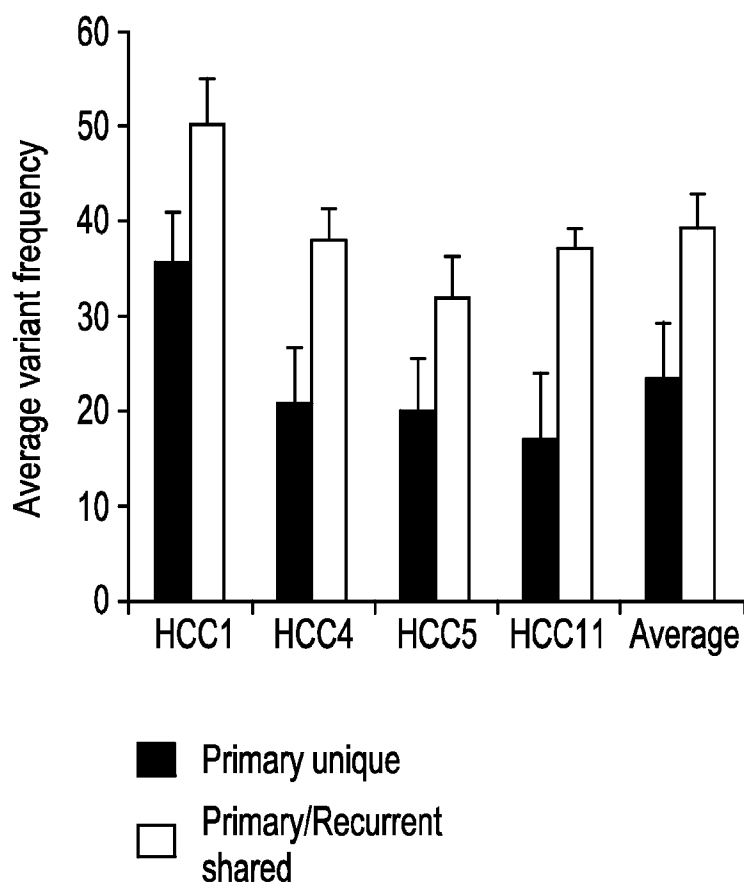
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FIG. 4



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FIG. 5



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FIG. 6A

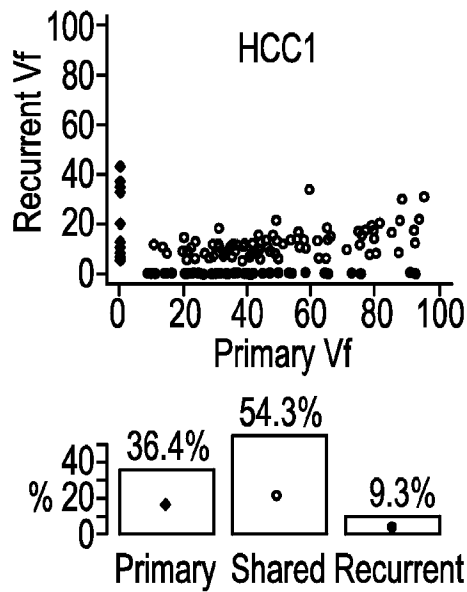


FIG. 6B

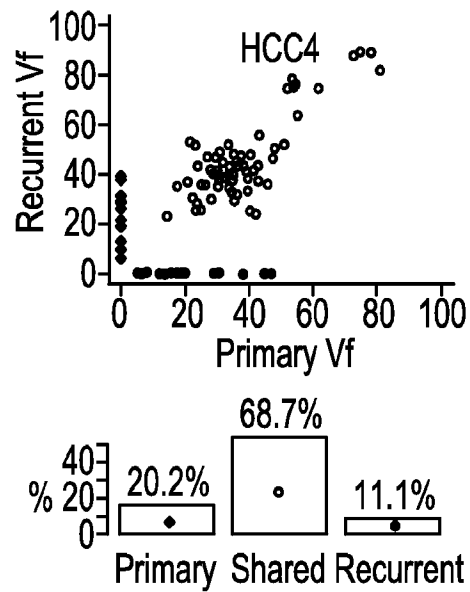


FIG. 6C

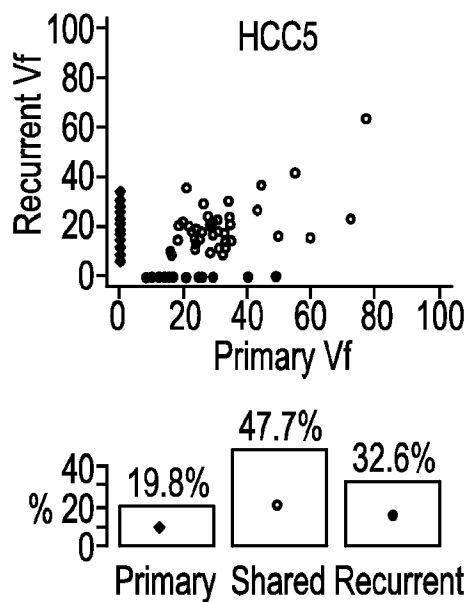


FIG. 6D

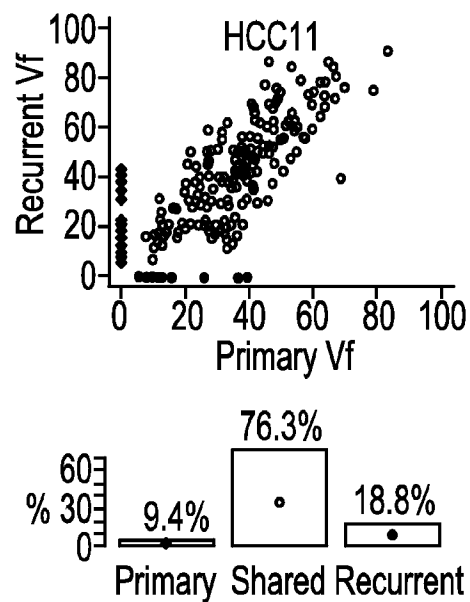
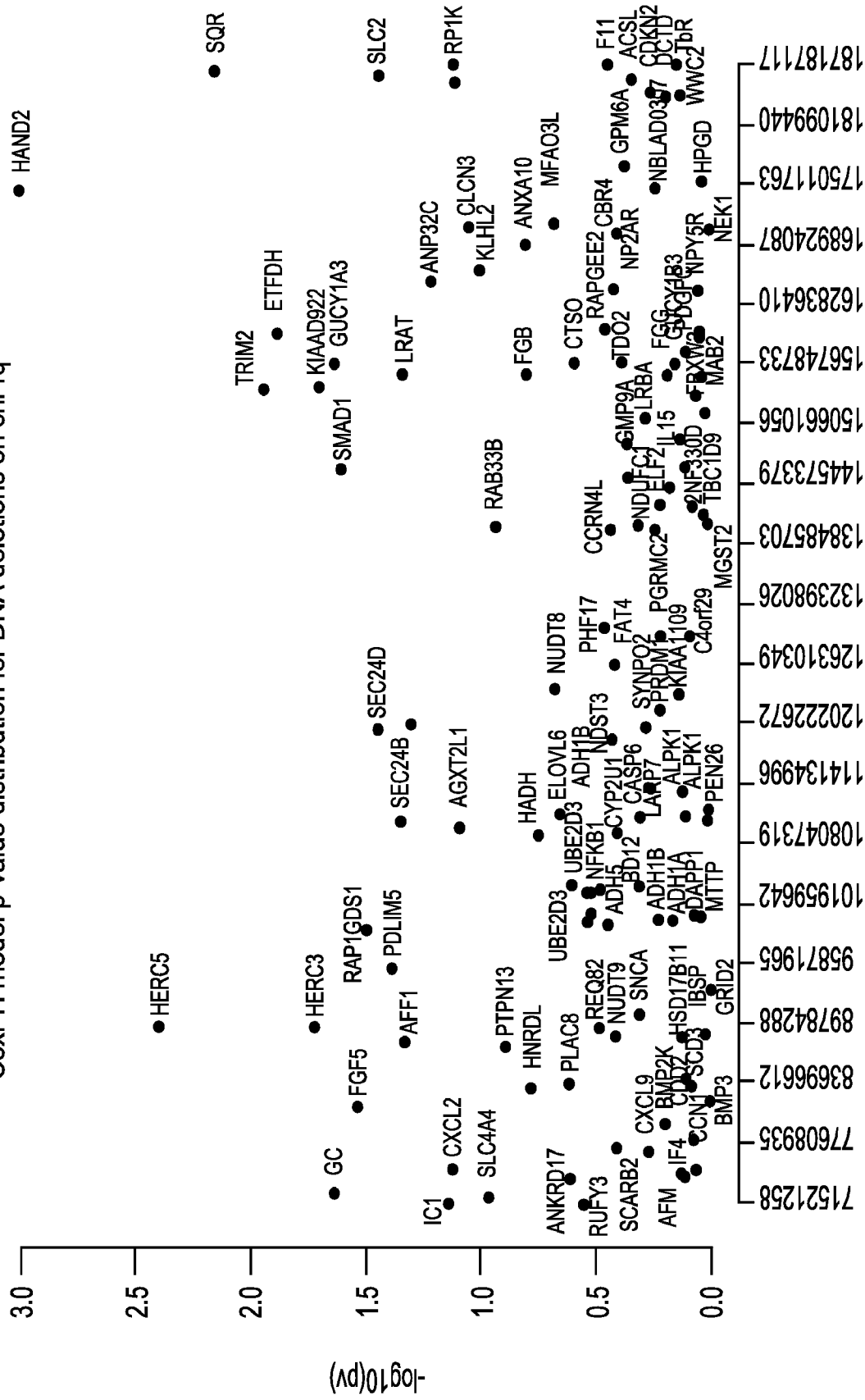


FIG. 7

CoxPH model p-value distribution for DNA deletions on chr4q



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FIG. 8A

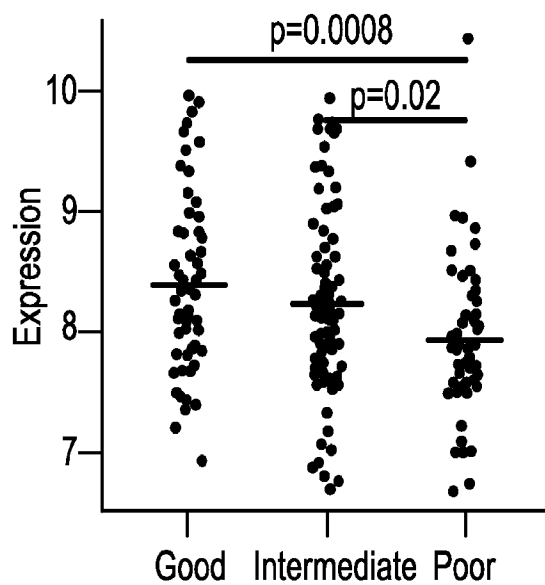
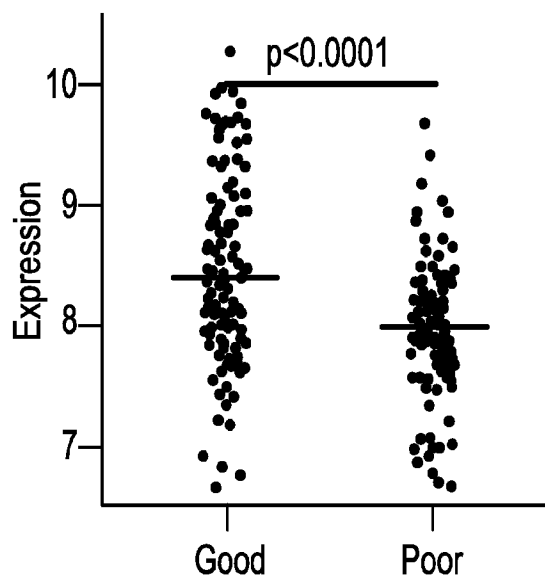


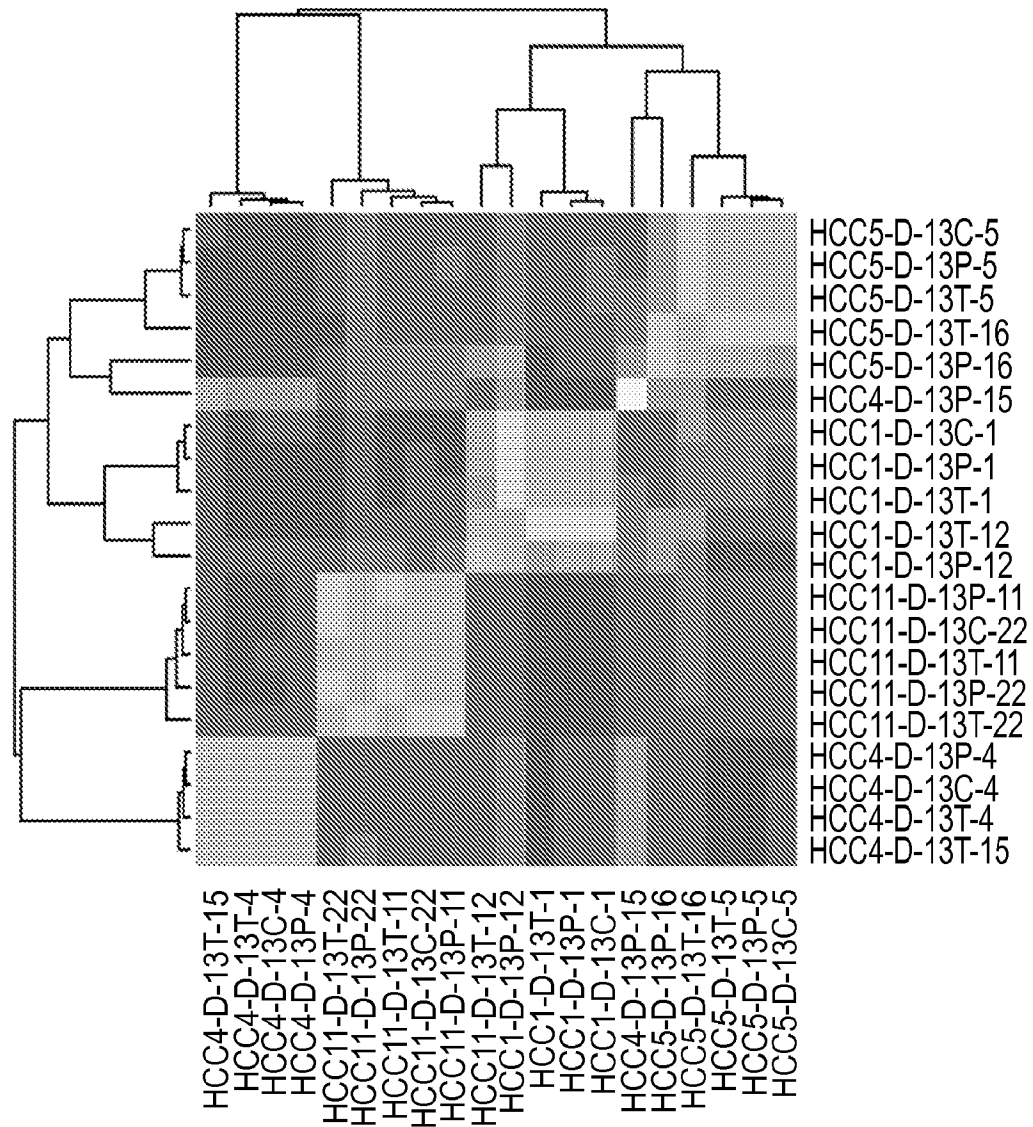
FIG. 8B



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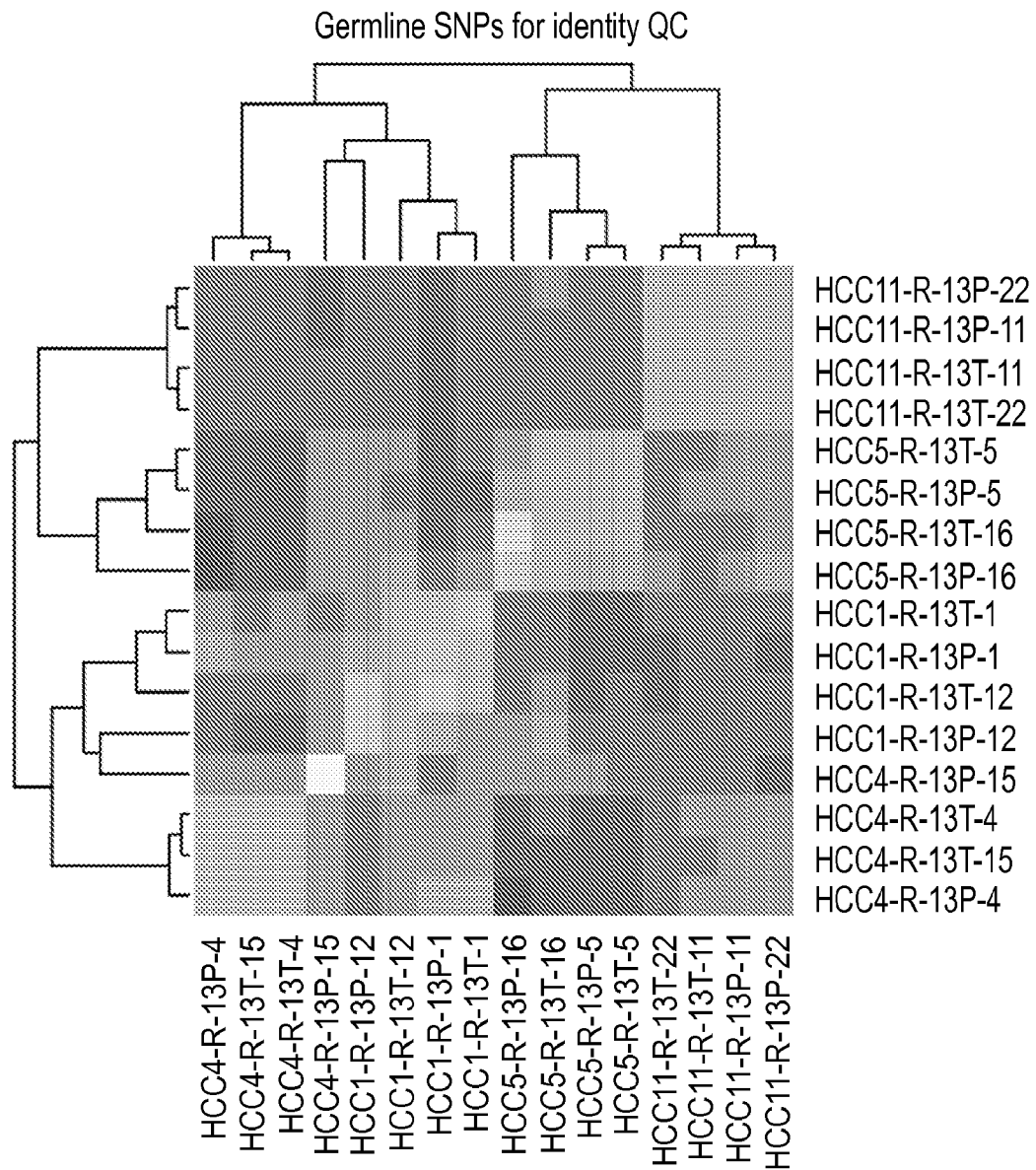
FIG. 9A

Germline SNPs for identity QC



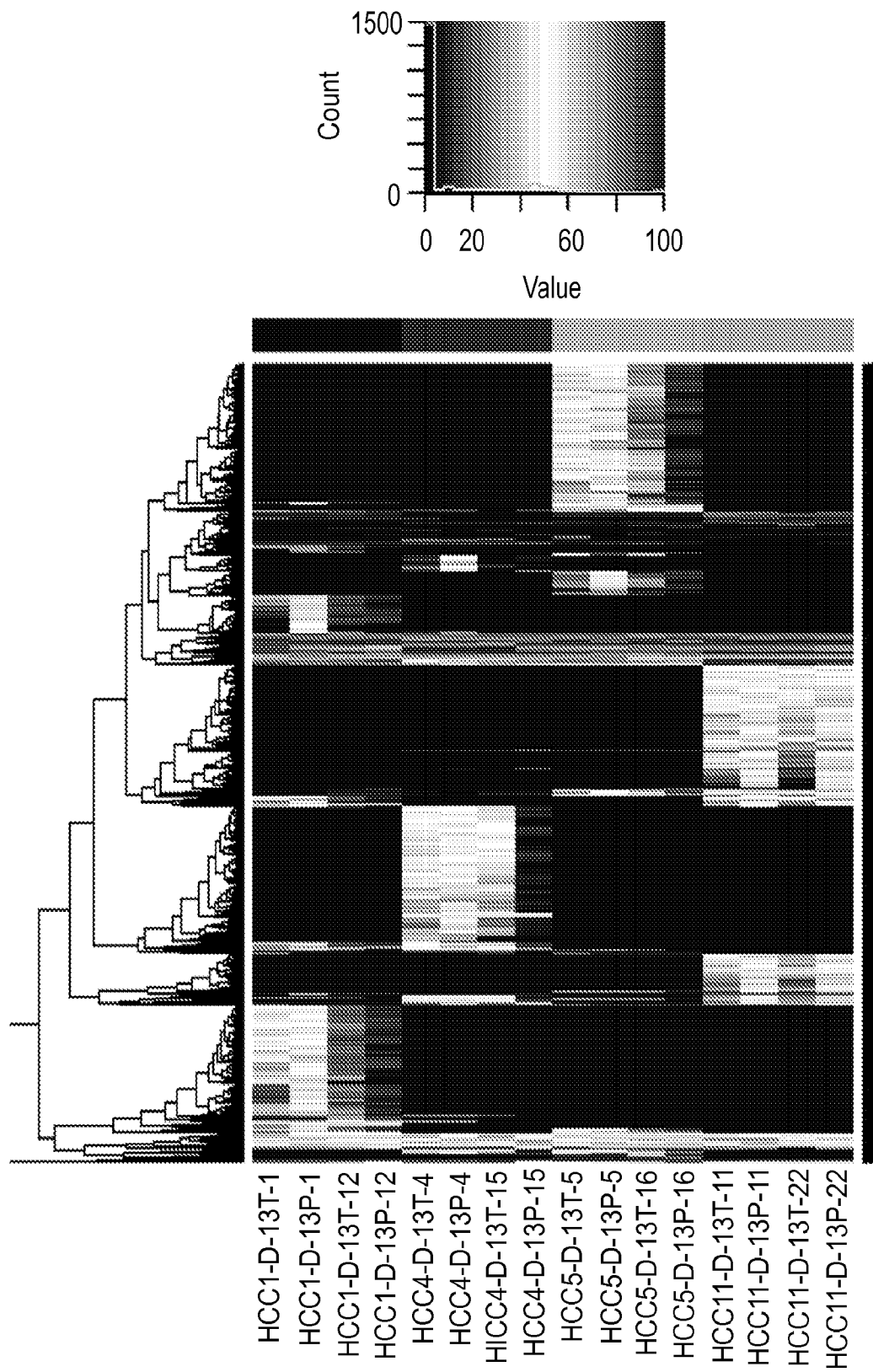
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FIG. 9B



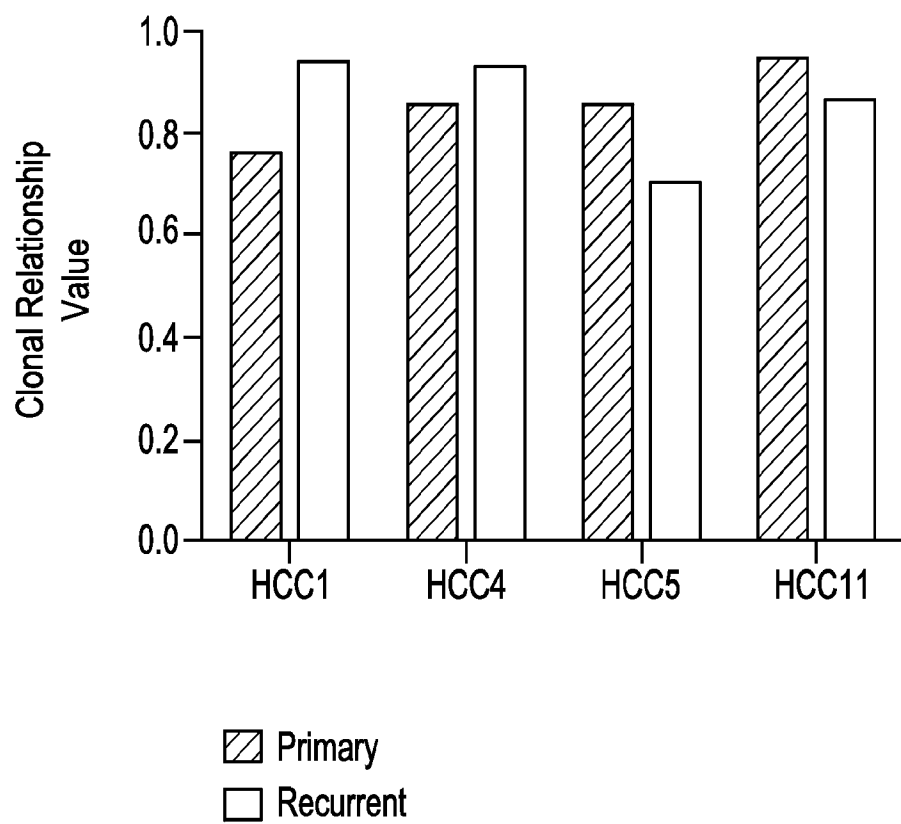
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FIG. 10



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FIG. 11



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FIG. 12A

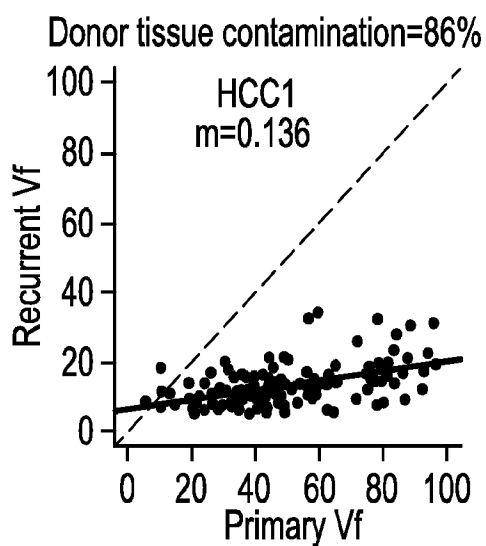


FIG. 12B

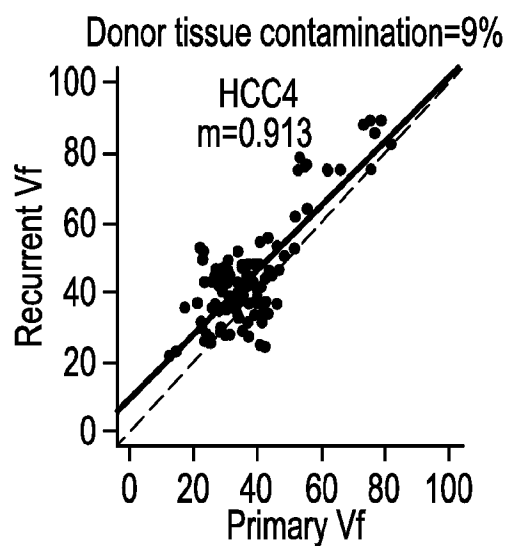


FIG. 12C

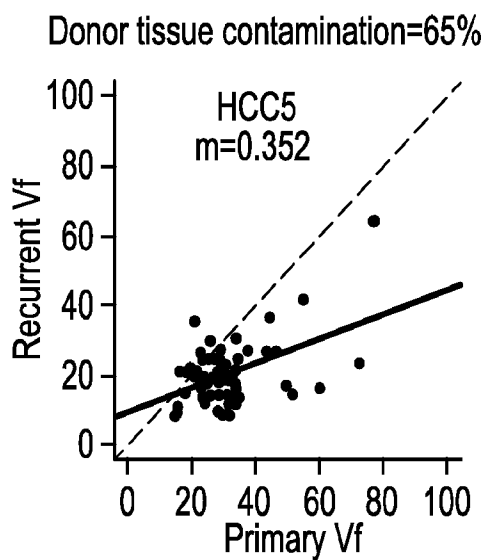


FIG. 12D

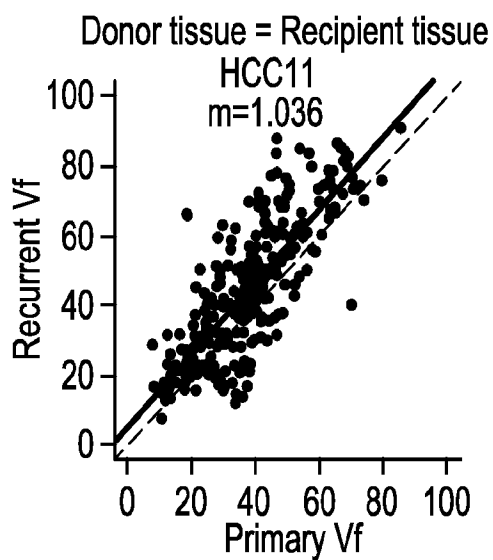
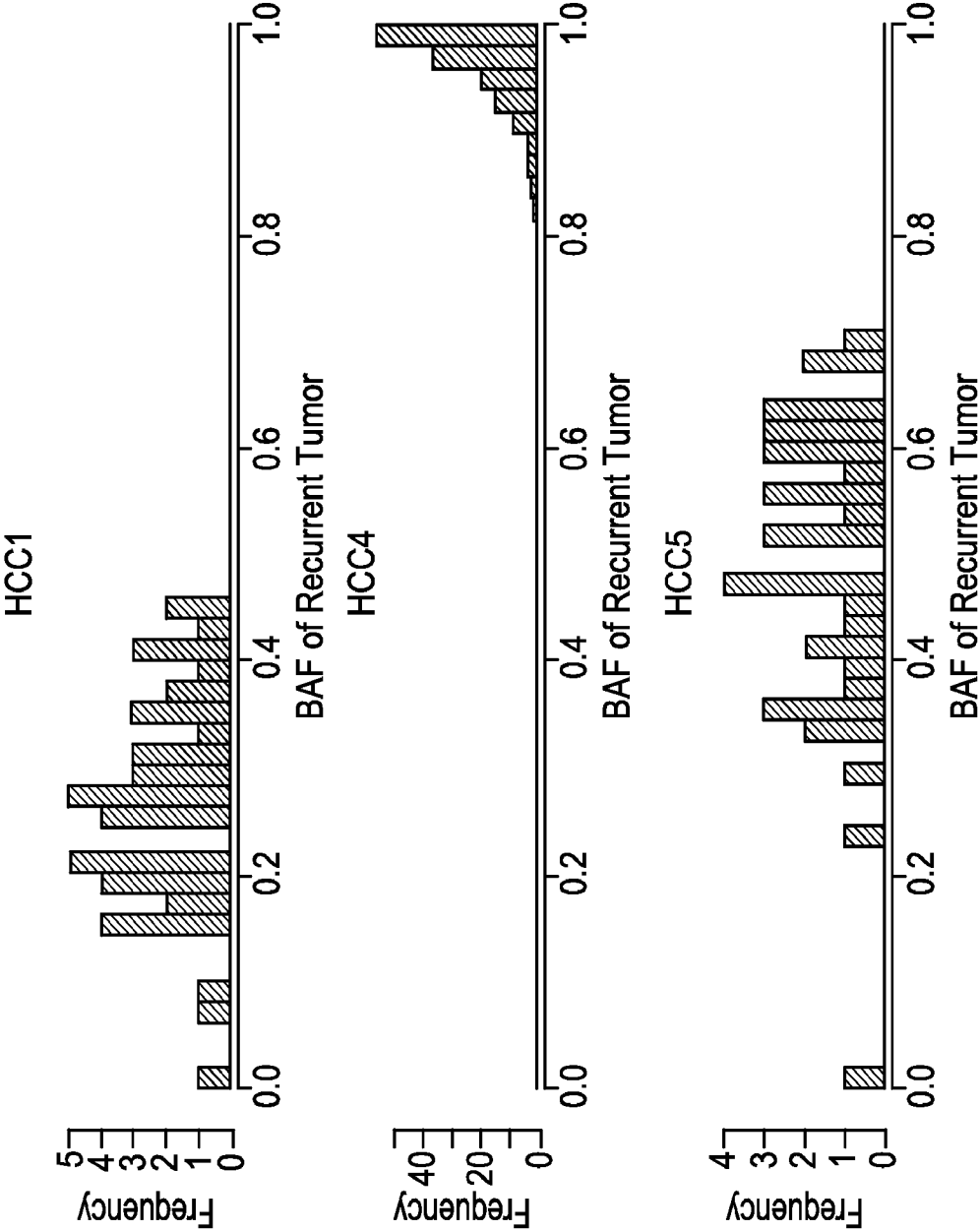


FIG. 13



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/16652

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68; C07H 21/04 (2015.01)

CPC - C12N 2310/322, 2310/315; A61K 31/7105

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12Q 1/68; C07H 21/04; CPC: C12N 2310/322, 2310/315; A61K 31/7105; USPC: 536/23.1, 22.1, 18.7, 1.11; 435/6.14, 6.1; 4, 91.1, 89, 85, 84, 72, 41

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer; Google; Google Scholar; Dialog ProQuest; Entrez PubMed; EBSCO; 'hepatocellular carcinoma,' 'HERC5,' 'orthotopic liver transplant,' 'mRNA,' expression, samples, 'recurrent carcinoma samples,' in vitro, primer, probe

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/118806 A2 (REGENTS OF THE UNIVERSITY OF CALIFORNIA) September 07, 2012; page 1, lines 14-20; page 2, line 20 -- page 3, line 15; page 4, lines 5-11; page 5, lines 9-20; page 36, lines 9-17; page 38, lines 7-18; page 42, lines 14-16; page 44, lines 17-22; page 56, lines 9-17	1-10, 14-17, 32, 47/45, 47/46, 48/45, 48/46, 60-67
Y	US 2012/0009148 A1 (SMITH, Y) January 12, 2012; paragraphs [0015], [0059], [0083], [0109], [0178], [0334]	1-10, 14-17, 31, 32, 45-48, 63-66, 67/65, 67/66
Y	WO 2007/058623 A1 (SINGAPORE HEALTH SERVICES PTE, LTD.) May 24, 2007; paragraphs [0003], [0015], [0017], [0024], [0040], [0041], [0055], [0064]; figure 3	4, 5/4, 6/4, 7-10, 16/14, 16/15, 17/14, 17/15, 31, 32, 45-48, 63-66, 67/65, 67/66
Y	WO 2013/101771 A2 (GENENTECH, INC.) July 04, 2013; paragraphs [0014], [0049], [0053], [0102], [0107], [0450]-[0452]	5/3, 5/4, 6/3, 6/4, 8, 16/14, 16/15, 17/14, 17/15, 47/45, 47/46, 48/45, 48/46, 60-62, 67/65, 67/66

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

01 June 2015 (01.06.2015)

Date of mailing of the international search report

26 JUN 2015

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US15/16652

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11-13, 18-30, 33-44, 49-59
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.