



- (51) **International Patent Classification:**
A61K 31/7105 (2006.01) *G01N 33/574* (2006.01)
C12Q 1/68 (2006.01)
- (21) **International Application Number:**
PCT/SG2015/050229
- (22) **International Filing Date:**
23 July 2015 (23.07.2015)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
10201404339X 23 July 2014 (23.07.2014) SG
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

(54) **Title:** HEPATOCELLULAR CARCINOMA PROFILING AND/OR THERAPY

(57) **Abstract:** The present invention relates to a method for profiling hepatocellular carcinoma comprising classifying survivin gene expression levels and/or survivin protein phosphorylation in HCC compared to a control. The present invention also includes treating HCC with survivin inhibitors, particularly in subjects with high survivin gene expression and/or survivin protein phosphorylation compared to normal liver tissue.



HEPATOCELLULAR CARCINOMA PROFILING AND/OR THERAPY

Field of the invention

The present invention relates to the field of profiling (in particular molecular profiling) and/or therapy of tumour.

5 Background of the invention

Hepatocellular carcinoma (HCC) has been the second leading cause of cancer-related deaths worldwide and tumours in most HCC patients are inherently resistant to many conventional chemotherapeutic drugs (Maluccio and Covey, 2012). Despite current development of molecular targeted therapies (Wörms and
10 Galle 2014) and immunotherapy (Sprinzl and Balle 2013), systemic chemotherapy for advanced HCC remains an urgent unmet medical need. Sorafenib is currently the only FDA-approved molecular inhibitor for the systemic therapy of advanced HCC. Although data from the Sorafenib Hepatocellular Carcinoma Assessment Randomised Protocol (SHARP) trial
15 (Llovet *et al.*, 2008) and the Asia-Pacific study (Cheng *et al.*, 2009) showed a significant survival benefit, the absolute gain in life expectancy was not impressive (10.7 vs. 7.9 months with placebo). Despite many ongoing clinical trials aim to improve the non-surgical treatment of HCC, the molecular therapy in HCC have been disappointing and over 80% of the phase 3 trials have been
20 failed in the last 5 years (Villanueva *et al.*, 2013).

It is therefore desirable to identify new therapeutic targets and develop drugs effective for treating HCC.

Summary of the invention

According to a first aspect, the present invention provides a method for profiling

5 a liver sample from a hepatocellular carcinoma (HCC) subject comprising:

- (i) determining survivin gene expression level and/or survivin protein phosphorylation in the liver sample;
- (ii) comparing the survivin gene expression level and/or survivin protein phosphorylation in the liver sample with survivin gene expression
10 level(s) and/or survivin protein phosphorylation, respectively from at least one control; and
- (iii) classifying the liver sample as either having higher, lower or equal survivin gene expression level and/or survivin protein phosphorylation compared to the control.

15 According to another aspect, the present invention relates to a method for monitoring a HCC subject comprising determining survivin gene expression levels and/or survivin protein phosphorylation of (a) at least one liver sample taken from the subject at a first time point before the subject has been administered at least one survivin inhibitor and (b) at least one liver sample
20 separately taken from the subject taken at various subsequent time points after the subject has been administered the at least one survivin inhibitor, wherein:

- (i) lower survivin gene expression level and/or survivin protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding positively to the survivin inhibitor;
- 5 (ii) higher survivin gene expression level and/or survivin protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding poorly to the survivin inhibitor; and
- (iii) substantially the same survivin gene expression level and/or survivin
10 protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding time point is indicative that the HCC subject is not responding to the survivin inhibitor.

The present invention further provides a method for treating HCC in a subject comprising administering at least one survivin inhibitor to the subject.

- 15 The present invention further includes a method for treating HCC comprising:
- (i) determining survivin gene expression level and/or survivin protein phosphorylation in a liver sample from a subject;
 - (ii) comparing the survivin gene expression level and/or survivin protein phosphorylation in the HCC sample with survivin gene expression
20 level(s) and/or survivin protein phosphorylation, respectively from at least one control; and

- (iii) administering at least one survivin inhibitor to a subject classified as having a higher survivin gene expression level and/or survivin protein phosphorylation compared to the control.

According to another aspect, the present invention relates to a survivin inhibitor
5 for use in treating HCC in a subject.

The present invention also relates to use of a survivin inhibitor in the preparation of a medicament for treating HCC in a subject.

Brief description of the figures

Figure 1 shows the remarkable heterogeneous expression of survivin in HCC
10 tissues (A) Expression of BIRC5 was shown by dot plot analysis, by searching the HCC Gene Expression database established in our laboratory using Affymetrix Human Genome U133 plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) comprising of HCC tumour and adjacent histologically normal liver tissues. (B) Expression of BIRC5 was associated with the disease-free survival
15 of patients with HCC. The median expression value obtained for BIRC5 of the samples was chosen as the cut-off point for survival analysis using the Kaplan-Meier method. (C) Validation of the expression of BIRC5 in another 40 pairs of HCC tumour and matched normal tissue samples as well as 10 cases of normal liver tissues by RT-qPCR. (D) The image of tissue array IHC staining for
20 validation the expression of survivin in another panel of HCC tumour tissues. (E) The imaging analysis and quantification of tissue array staining with survivin.

The 60 spots in the tissue array slide were from 30 patients' HCC samples and duplicate spots for each patient. The IHC images were evaluated according to the percentage of cells with positive nuclei.

Figure 2 shows the expression of survivin (BIRC5) in the HCC samples reported by Roessler *et al.*, 2012. (A) Expression of survivin was shown by dot plot analysis after searching the Gene Expression database available in GEO (gse14520_raw). (B) The ID number of HCC samples for IHC staining in a tissue array to validate the expression of survivin in an independent panel of HCC tumour tissues.

Figure 3 shows the heterogeneity expression and phosphorylation status of survivin affects sensitivity to the survivin suppressant YM155 in HCC cells. (A) The expression and phosphorylation status of survivin protein in a panel of HCC patients tumor (T) and matched normal (N) tissue samples by western blotting analysis. (B) The expression and phosphorylation status of survivin protein in a panel of liver cancer cell lines and two normal liver tissues by western blotting analysis.

Figure 4 shows that YM155 inhibits cell growth in sensitive liver cancer cells. (A) Incubation of HCC cell lines in YM155 for (i) 24h and (ii) 48h at different concentrations; (B) Incubation of HCC cell lines in sorafenib for (i) 24h and (ii) 48h at different concentrations. (C) Western blot analysis of Mahlavu cells after incubating for either 24h or 48h in 10ng/ml (C) and 48h treatment 1 ng/ml or 10

ng/ml of YM155 (D). Figure 5 shows that YM155 inhibits the anchorage-independent cell growth, induced cell cycle arrest, apoptosis and DNA damage of high survivin-expressing sensitive HCC cells. Representative image (A) and statistical analysis (B) of the inhibition effect of YM155 on anchorage-independent cell growth of sensitive Mahlavu and HLE HCC cells (* $P < 0.05$, ** $P < 0.01$). Representative image (C) and statistical analysis (D) of the inhibition effect of YM155 on anchorage-independent cell growth of resistant HepG2 and HuH7 HCC cells. (E) Representative images of the effect of YM155 on cell cycle progression. The G2/M phase arrest was observed in both Mahlavu and HLE cells treated with YM155. (F) The increase in Annexin-V+ cells was observed by flow cytometry analysis in both Mahlavu and HLE cells treated with YM155. (G) The increasing of TUNEL+ cells was observed by confocal microscopy analysis in both Mahlavu and HLE cells treated with YM155. (H) Increase in cleaved Caspase 3 and PARP, P-H2AX could be detected by Western blot following treatment of Mahlavu cells with increasing doses of YM155. (I) Mahlavu and HLE cells were treated with 10ng/ml YM155 and stained for P-H2AX and Hoechst 33342 and analysed by confocal microscopy. The green signal represents staining for P-H2AX. Nuclear DNA was detected by staining with Hoechst 33342.

Figure 6 shows effects of YM155 on the apoptosis and DNA damage of HuH7 and HepG2 cells. (A) The represent images of TUNEL staining. There were few TUNEL+ cells observed by confocal microscopy analysis in both HuH7 and

HepG2 cells treated with 10ng/ml YM155. The green signal represents staining for TUNEL+ cells. Nuclear DNA was detected by staining with Hoechst 33342.

(B) The represent images of DNA damage marker p-H2AX staining. The HuH7 and HepG2 cells were treated with 10ng/ml YM155 and stained for p-H2AX and Hoechst 33342 before being analysed by confocal microscopy. The green signal represents staining for p-H2AX. Nuclear DNA was detected by staining with Hoechst 33342.

Figure 7 shows that knockdown survivin decreased the cell sensitivity to YM155 and the possible underlying molecular mechanisms involved in the induction of cell death by YM155. (A) The knockdown of survivin by siRNA transfection using qRT-PCR analysis. (C and D) Ingenuity Pathway Analysis using the differentially expressed genes identified using microarrays. (C) 10ng/ml YM155-treated Mahlavu cells and control Mahlavu cells, or (D) Survivin siRNA (50nM)-treated Mahlavu cells and control Mahlavu cells using 1.5-fold in differential expression to generate the gene list to construct the networks. (E) Effects of YM155 and survivin knockdown on the cell cycle G2/M DNA damage checkpoint regulatory-related genes (CCNB1, CKS2, WEE1, API5 and GADD45A) using real time RT-qPCR analysis.

Figure 8 shows that YM155 is a promising therapeutic agent targeting HCC with high survivin expression in the xenograft models of established HCC cells. (A and C) The representative orthotopic mouse liver tumour xenograft model images (A: Mahlavu-luciferase cells mice model, B: HuH7-luciferase cells mice

model) from each group were shown for each week of different treatments. (B and D) Quantitative analysis of bioluminescence imaging signals of all mice during each week of different treatments is shown. (E and F) The expression of survivin in tumor tissues of Mahlavu (E) and HuH7 (F) cells by immunohistochemical analysis and the inducing of tumor cell apoptosis (green TUNEL+ cells) in Mahlavu (E) and HuH7 (F) tumor by TUNEL staining.

Figure 9 shows that YM155 is a promising therapeutic agent targeting HCC with high survivin expression in the patient-derived xenograft expressing high survivin. (A) The expression level of Survivin in three patient-derived HCC tissue samples by western blotting analysis. (B) Quantitative analysis of tumour volume of all mice in each week of different treatments is shown. (C) The treatment endpoint image of SCID mice xenograft models using P3 patient-derived HCC tissue samples. (D) The treatment end point image of tumour size. (E) The expression of survivin in tumor tissue by immunohistochemical analysis and the increasing of tumor cell apoptosis (green TUNEL+ cells) by TUNEL staining.

Figure 10 shows the expression of SLC35F2 in our HCC dataset (A) and in the published HCC dataset of Roessler *et al.*, (2012)

Definitions

As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings.

10 A subject with HCC is one diagnosed with HCC based on the typical diagnostic criteria for HCC.

A healthy individual not suffering from HCC is assessed as HCC negative based on the typical diagnostic criteria for HCC.

Abbreviations: HCC, hepatocellular carcinoma; BIRC5, survivin, also called
15 baculoviral inhibitor of apoptosis repeat-containing 5; IAPs, inhibitor-of-apoptosis proteins; SHARP, Sorafenib Hepatocellular Carcinoma Assessment Randomised Protocol; SCID, Severe combined immunodeficiency; EGFR, epidermal growth factor receptor; TKIs, tyrosine kinase inhibitors; NSCLC, non-small-cell lung carcinoma; HER2, Human Epidermal Growth Factor Receptor 2;
20 DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MTS, 3-(4,5-di methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium; HPRT1, hypoxanthine phosphoribosyltransferase 1; RIPA, radioimmunoprecipitation; CCNB1, cyclin B1; PARP-1, Poly (ADP-ribose) polymerase 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IHC, Immunohistochemistry; TUNEL, Terminal deoxynucleotidyl transferase dUTP
5 nick end labelling; CIRB, Centralised Institutional Review Board; IACUC, Institutional Animal Care and Use Committee; IPA, Ingenuity Pathway Analysis; EC50, concentration for 50% of maximal effect; H2AX, H2A histone family, member X (H2AX becomes phosphorylated on serine 139, then called γ H2AX).

Detailed description of the invention

- 10 According to a first aspect, the present invention provides a method for profiling a liver sample from a hepatocellular carcinoma (HCC) subject comprising:
- (i) determining survivin gene expression level and/or survivin protein phosphorylation in the liver sample;
 - (ii) comparing the survivin gene expression level and/or survivin protein
15 phosphorylation in the liver sample with survivin gene expression level(s) and/or survivin protein phosphorylation, respectively from at least one control; and
 - (iii) classifying the liver sample as either having higher, lower or equal gene
20 expression level and/or survivin protein phosphorylation compared to the control.

According to another aspect, the present invention relates to a method for monitoring a HCC subject comprising determining survivin gene expression

levels and/or survivin protein phosphorylation of (a) at least one liver sample taken from the subject at a first time point before the subject has been administered at least one survivin inhibitor and (b) at least one liver sample separately taken from the subject taken at various subsequent time points after

5 the subject has been administered the at least one survivin inhibitor, wherein:

- (i) lower survivin gene expression level and/or survivin protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding positively to the survivin inhibitor;
- 10 (ii) higher survivin gene expression level and/or survivin protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding poorly to the survivin inhibitor; and
- (iii) substantially the same survivin gene expression level and/or survivin
15 protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding time point is indicative that the HCC subject is not responding to the survivin inhibitor.

The liver sample from the HCC subject may be diseased liver tissue from the subject. It will be appreciated that the diseased liver tissue comprises
20 transformed cells. It will be further appreciated that the liver sample may be taken from a liver neoplasm.

It will be appreciated that any suitable control may be used. Typically, the control comprises at least one normal liver tissue sample. A normal liver tissue sample may be from a healthy individual not suffering from HCC. A normal liver tissue sample may also be from non-diseased liver tissue from a subject with
5 HCC. Non-diseased liver tissue refers to liver tissue with non-transformed cells. Typically, non-diseased liver tissue and/or cells can be easily distinguished from the HCC tissue, for example morphologically. It will be appreciated that non-diseased liver tissue and/or cells also do not show any manifestation of any other liver conditions, including but not limited to liver cirrhosis, hepatic steatosis
10 and hepatitis, for example.

For the first aspect of the invention, it may also be informative to classify a liver sample from a HCC subject by comparing with a control also comprising at least one liver sample from at least one HCC subject rather than a normal liver tissue sample. For example, control HCC subjects may be diagnosed as early stage,
15 intermediate stage or advanced stage HCC.

For the first aspect of the invention wherein the liver sample is taken from a liver neoplasm and the control comprises at least one normal liver tissue sample, it will be appreciated that the method further comprises identifying a subject with higher expression of survivin gene and/or survivin protein phosphorylation
20 compared to the control as likely to have a higher recurrence of hepatocellular carcinoma.

It will be appreciated that the method for profiling a liver sample from a HCC subject or the method for monitoring a HCC subject as described herein may be an *in vitro* method.

The present invention further provides a method for treating HCC in a subject
5 comprising administering at least one survivin inhibitor to the subject.

The present invention further includes a method for treating HCC comprising:

- (i) determining survivin gene expression level and/or survivin protein phosphorylation in a HCC sample from a subject;
- (ii) comparing the survivin gene expression level and/or survivin protein
10 phosphorylation in the HCC sample with survivin gene expression level(s) and/or survivin protein phosphorylation from at least one control; and
- (iii) administering at least one survivin inhibitor to a subject classified as having a higher survivin gene expression level compared to the control.

15 It will be appreciated that survivin gene expression level(s) may be determined at the transcription and/or translation level(s).

Any suitable method may be employed for determining survivin gene expression level(s) at the transcription level, including but not limited to Northern blot analysis, microarray analysis and/or reverse-transcription PCR. In

particular, the reverse-transcription PCR may be quantitative reverse-transcription PCR (RT-qPCR).

Any suitable method may also be used to determine survivin gene expression level at the translation level and/or survivin protein phosphorylation, including
5 but is not limited to Western blot, immunohistochemistry (IHC) and/or enzyme-linked enzyme-linked immunosorbent assay (ELISA) analysis.

According to another aspect, the present invention relates to a survivin inhibitor for use in treating HCC in a subject. The present invention also relates to use of a survivin inhibitor in the preparation of a medicament for treating HCC in a
10 subject. In particular, a HCC sample from said subject has higher survivin protein expression and phosphorylation compared to at least one normal liver tissue sample and further, it will be appreciated that said subject will be profiled by a method as described herein.

Any suitable survivin inhibitor is applicable for relevant aspects of the invention
15 as described herein. The survivin inhibitor may inhibit survivin gene expression. For example, the survivin inhibitor comprises at least one RNA interfering agent targeting survivin mRNA. The RNA interfering agent may comprise a small interfering RNA.

Alternatively, the survivin inhibitor may inhibit survivin protein activity and/or
20 survivin phosphorylation. Examples of survivin inhibitor includes but is not limited to 1-(2-methoxyethyl)-2-

5 methyl-3-(pyrazin-2-ylmethyl)-2H-benzo[f]benzimidazole-4,9-dione;bromide (YM155). Another example of a survivin inhibitor may be at least one antibody and/or a functional fragment thereof. The at least one antibody may be a monoclonal antibody or polyclonal antibodies. Monoclonal and polyclonal antibodies may be produced by standard methods. A further example of a survivin inhibitor includes a peptide.

10 It will be appreciated that the present invention is suitable for personalised treatment, for example a subject is profiled for survivin gene expression levels and survivin protein phosphorylation to assess response to survivin inhibitor before administration of treatment.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

15 Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Green (2012).

Example 1 Materials and Methods

20 From screening hundreds of compounds in a panel of liver cancer cells to identify effective therapeutic drugs, it was interestingly found that YM155, a survivin inhibitor, was shown as a promising therapeutic agent against HCC with survivin over-expression. The heterogeneity in expression of survivin was monitored in a panel of liver cancer cells and tissues. The orthotopic xenograft

models and patients' derived HCC xenografts were employed to monitor the therapeutic effects of YM155 *in vivo*.

Tissue specimens, cell lines and reagents

5 The collection of tumor and adjacent normal liver tissues from HCC patients were approved by the National Cancer Centre Institutional Review Board (IRB) and all tissues studied were provided by the Tissue Repository of the National Cancer Centre Singapore (NCCS) and Cancer Center, Sun Yat-Sen University. Written informed consent was obtained from all participating patients and all
10 clinical and histopathological data provided to the researchers were rendered anonymous. Liver cancer cells were cultured in Dulbecco's modified Eagle's medium (HepG2, HuH7, PLC/PRF/5, HCCLM3, HLE, Mahlavu, SNU-449, SK-HEP-1), with 10% FBS and 100 units/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen, Carlsbad, CA). All cell lines were maintained at 37°C
15 in the presence of 5% CO₂. Sorafenib was from Bayer HealthCare Pharmaceuticals, Inc. and YM155 was synthesised by MedChemexpress Co. Ltd and Biochempartner Co. Ltd (Shanghai, China).

Cell viability

The cell viability was assessed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetra zolium] assays using the
20 CellTiter 96 AQueous One Solution Cell Proliferation Assay kit from Promega

following the manufacturer's instructions. Each experiment was repeated three times.

RNA extraction, microarray and RT-qPCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) and the quality and
5 quantity of isolated total RNA was assessed by the Agilent 2100 Bioanalyzer
and NanoDrop ND-1000 Spectrophotometer (Agilent, Santa Clara, CA, USA).
The microarray and *RT-qPCR analysis* were performed as described (Wang *et al.*, 2011). Microarray analysis was performed as described (Wang *et al.*, 2011)
using the GeneChip® Human Gene ST Arrays (Affymetrix, USA) according to
10 the manufacturers' instructions. Post hybridization washes were performed on
an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an
Affymetrix GeneChip Scanner 3000. Scanned arrays were normalized using
GCRMA in Partek software (Partek Incorporated, St. Louis, MO). Array quality
control was performed using Affymetrix® Expression Console™. Signal
15 intensities were transformed to log2 base and imported to Partek Genomics
Suite software (Partek Inc., St. Louis, MO) to conduct statistical analyses. The
microarray data have been deposited in the European Bioinformatics Institutes
of the European Molecular Biology Laboratory database
(<http://www.ebi.ac.uk/array-express/>) and are accessible through ArrayExpress
20 public database with accession numbers E-MEXP-84 and E-TABM-292. The
microarray data were further analysed using Ingenuity Pathway Analysis (IPA)
Software. For mRNA detection by qRT-PCR, the total RNA was reversely

transcribed by using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, CA). qPCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) with hypoxanthine phosphoribosyltransferase 1 (HPRT1) as an internal control as described previously, and fold changes were calculated via relative quantification ($2^{-\Delta Ct}$).

Western blotting

Protein was isolated using RIPA. Antibodies used for western blotting were: rabbit anti-survivin (#2808) or Phospho-Survivin (Thr34) (D2E11) Rabbit mAb(#8888) (Cell Signaling, MA); mouse anti-PARP-1 and Caspase-3 (Santa Cruz, CA) and goat GAPDH (GenScript, NJ).

Immunohistochemistry (IHC)

The IHC was performed as described previously. Primary antibodies used included the rabbit anti-Survivin (#2808) (Cell Signaling, MA) (1:100), or an isotype-matched IgG as a negative control. Intensity of staining was evaluated based on a scale of 0 to 4 according to the percentage of positive tumours (0, negative control; +, 0%-10%; ++, 10%-25%; +++, 25%-50% and +++, >50%).

Immunofluorescence analysis

The HCC cells were seeded in the BD Falcon™ 8-well CultureSlide and treated with YM155 (10µg/ml) for 24 hours. The treated cells were fixed and incubated with primary antibodies against p-H2AX and then incubated with Alexa Fluor®

488 goat anti-rabbit IgG (Invitrogen). Slides were counterstained with Hoechst 33342 and imaged using a confocal laser-scanning microscope (Carl Zeiss).

Flow-cytometry

The cell cycle and apoptosis was analysed by flow cytometry (FACSCanto II, BD Biosciences) using PI staining or Annexin V/7-AAD kits (BD Biosciences) according to the standard protocol.

TUNEL assay

For labelling the nuclei of apoptotic cells, HCC cells were plated on glass coverslips in 24-well plates and fixed in 4% paraformaldehyde 24 hours post-YM155 treatment. TUNEL staining was performed using the DeadEnd fluorometric TUNEL system (Promega) according to the manufacturer's protocol. The number of TUNEL-positive cells was divided by the number of Hoechst 33342- stained cells to calculate the percentage of apoptotic nuclei.

Clonogenicity assay

Cells were plated in 6-well plates and treated with YM155 (1µg/ml or 10µg/ml) in culture medium. Upon the appearance of clones, the cells were fixed in methanol for 3 minutes and stained with a 0.01% crystal violet solution to assess colony formation. The number of macroscopically detectable colonies was registered. Treatments were performed in duplicate.

Animal studies

All experiments were approved by the Institutional Animal Care and Use Committee of SingHealth. All mice studies were done in the Animal Unit with AAALAC accreditation in National Cancer Center Singapore. Mahlavu- and HuH7-luciferase expressing cells were firstly subcutaneously injected into 5- to 6-week-old athymic mice (2 mice per group, BioLASCO Taiwan Co, Ltd) to generate tumour. The tumour-bearing mice were anesthetized with Hypnorm/Midazolam and a small piece of tumour was harvested and orthotopically transplanted to the liver of the recipient mice as described previously (Ong *et al.*, 2013) to establish the orthotopic mouse liver tumor xenograft model. One week after implantation, the mice were imaged and grouped. Mice with similar signals were grouped (10 mice/group) and treated either with YM155, sorafenib or saline for 7 weeks. Sorafenib was administered at the effective dose of 30 mg/kg/dose, p.o. Mice were treated with YM155 for 7 days with a continuous intraperitoneal injection of 10 mg/kg, followed by a 7-day rest period. Each cycle was repeated every 14 days for a total of 4 cycles of treatment. Tumor growth was monitored every week by bioluminescence imaging using the Xenogen IVIS Lumina system (Xenogen Corporation, Hopkinton, MA).

Survival and statistical analysis

The experimental data are presented as the mean $\pm$ standard deviation (SD). All statistical analyses were performed using ANOVA or a two-tailed Student's *t* test (GraphPad Prism 5). The survival curves were created using the Kaplan-Meier method and statistically compared using a log-rank test. Differences were considered statistically significant when the P-values were less than 0.05.

Example 2 Results

The remarkable heterogeneous expression of survivin in HCC tissues

The expression of survivin is quiescent in most normal, terminally differentiated tissues but it is widely expressed in cancers, including HCC. A global gene expression database on human HCC tumour and adjacent histologically normal liver tissues using Affymetrix Human Genome U133 plus 2.0 Arrays (Wang *et al.*, 2011 and Xia *et al.*, 2013). To evaluate the potential role of survivin as a therapeutic target for HCC, the expression of survivin in our dataset was systematically examined. The increased expression of survivin was found to be remarkably heterogeneous in HCC, the detected signal intensity ranged from $\log_2 2$ to $\log_2 9$ (Figure 1A). Furthermore, when the survival analysis was performed with the median expression value of BIRC5 as the cut-off, high expression of survivin in HCC tissues was significantly associated with shorter disease-free survival in our dataset (Figure. 1B). The relationship between

BIRC5 expression level and clinicopathological parameters of HCC is shown in Table 1. This heterogeneous expression of survivin in HCC was also observed using the reported dataset of Roessler *et al.*, 2012 (Figure. 2A).

Table 1 Relationship between survivin expression and clinicopathological parameters of HCC

Patients' characteristics	Number of cases	Survivin expression		p-value
		Low	High	
Age (years)				
≤60	36	18	18	0.828
>60	40	21	19	
Sex				
M	66	36	30	0.148
F	10	3	7	
Tumour size (cm)				
≤3	23	14	9	0.272
>3	53	25	28	
AJCC tumour staging				
I	46	27	19	0.267
II	21	8	13	
IIIA	9	4	5	
AFP (ng)				
≤20	38	26	12	0.003
>20	38	13	25	
Tumour venous infiltration				
VI	28	12	16	0.26
NI	48	27	21	
Child's grade (A) (B)				
A	56	33	14	0.026
B	20	6	23	
No. of tumour nodules				
1	67	32	35	0.091
≥2	9	7	2	
Cirrhosis				
Yes	36	16	20	0.19
No	55	21	14	
Recurrence (Months)				
≤24	36	14	22	0.024
>24	35	23	12	

p-value < 0.05 indicates significance. VI: venous infiltration, NI: No infiltration

The heterogeneous expression of survivin was further validated in 40 pairs of HCC samples by real time RT-qPCR analysis (Figure 1C) and an additional independent cohort of 30 pairs of HCC samples by IHC (Figures 1D, 1E and 2B). The heterogeneous expression of survivin studied by IHC was quantitated in cells with positive nuclei staining. There are 24 out of 30 (80%) of HCC samples that yielded an increase expression of survivin. Among them, 18 out of 30 (60%) of HCC samples gave a 2-fold increase in the expression of survivin while 10 out of 30 (33.33%) of HCC samples gave a 5-fold increase in the expression of survivin (Figures 1D and 1E).

10 *The heterogeneity expression and phosphorylation status of survivin affects sensitivity to the survivin suppressant YM155 in HCC cells*

The observation that survivin is expressed in neoplastic tissues yet undetectable in most normal differentiated tissues makes it a promising therapeutic target in cancer chemotherapy. The regulatory process of survivin on apoptosis and cell cycle requires the phosphorylation of survivin at Thr34. The expression and phosphorylation of survivin in a panel of HCC tissues and liver cancer cells was examined by western blotting (Figures 3A and 3B). The heterogeneous expression and phosphorylation of survivin could also be demonstrated: expression of survivin was relatively high in the cell lines Hep3B, HLE and Mahlavu and comparatively lower in HepG2 and HuH7 cells (Figure 3B). Next, the chemosensitivity of these HCC cells to YM155, a survivin inhibitor, which had been screened from hundreds of compounds was

compared and it was observed that YM155 showed remarkable heterogeneity sensitivity to a panel of liver cancer cells. Interestingly, similarly to the heterogeneous expression of survivin in HCC cells, it was observed that liver cancer cells have different sensitivity to YM155 (Figure 4A. The observed concentration for 50% of maximal effect (EC₅₀) of YM155 for the high survivin-expressing cells Mahlavu and HLE was <10ng/ml. In comparison, the EC₅₀ of YM155 for the low survivin-expressing cells HuH7 and HepG2 was >1000ng/ml following 24h- or 48h-treatments (Figure. 4A (i) and (ii), respectively). This is in remarkable contrast to sorafenib, which gave an overall EC₅₀ >1000ng/ml for most of the HCC cells tested (Figure. 4B (i) and (ii)). It appeared that the sensitivity of liver cancer cells to YM155 correlated with their level of survivin expression and phosphorylation: high survivin-expressing cells, Mahlavu and HLE, are more sensitive than low survivin-expressing cells, HuH7 and HepG2.

YM155 inhibits survivin expression and anchorage-independent cell growth in sensitive liver cancer cells

Next, the time and dose dependent inhibition effects of YM155 on the sensitive liver cancer cells Mahalvu and HLE were evaluated. It was observed that 10ng/ml YM155 can significantly inhibit the survivin expression in Mahlavu cells at the time point of 24 h and 48 h treatment (Figure 4C). The YM155 can inhibit survivin expression in Mahlavu cells even at a low dose of 1ng/ml (Figure 4D). The ability of cancer cells to grow under anchorage-independent conditions is one of the hallmark properties that are associated with the tumorigenic potential

of a cancer cell. The effects of YM155 on the anchorage-independent cell growth ability were subsequently tested in the sensitive and resistant liver cancer cells. When Mahlavu and HLE cells were plated separately in soft agar and treated with YM155 (1ng/ml and 10ng/ml), both Mahlavu and HLE cells showed a statistically significant reduction in anchorage-independent growth at 1ng/ml and 10ng/ml YM155 versus medium control (Figures 5A and 5B), while treatment of HuH7 and HepG2 at both 1ng/ml and 10ng/ml of YM155 produced little effect (Figures 5C and 5D). Therefore, YM155 inhibits survivin expression and anchorage-independent cell growth in sensitive liver cancer cells, providing a promising therapeutic potential for liver cancer with high survivin expression. YM155 may potentially result in good clinical efficacy for treatment of HCC with high survivin expression.

YM155 induced cell cycle arrest, apoptosis and DNA damage in high survivin-expressing sensitive liver cancer cells

When YM155-sensitive Mahlavu and HLE cells were treated with 10ng/ml YM155 for 12h, significant cell cycle arrest in the G2/M phase was observed. The G2/M phase cell sub-population of the HLE cells increased approximately 7-fold and a 4-fold increase was observed in the Mahlavu cells (Figure 5E); however, the effect of YM155 on cell cycle arrest in YM155-resistant cells was not observed. Moreover, Annexin-V/7-AAD analysis and TUNEL assay demonstrated that TUNEL-positive cells were significantly increased for the YM155-sensitive Mahlavu and HLE cells (Figures 5F and 5G) compared to the

YM155-resistant HuH7 and HepG2 cells (Figure 6A) following 24h treatment with 10 ng/ml YM155. The increased induction of apoptosis in the Mahlavu cells with increased YM155 drug concentrations correlated well with the significant enhancement in caspase 3 and PARP cleavage (Figure 5H). To determine
5 whether YM155 stimulated a DNA damage response in the YM155-sensitive liver cancer cells, immunofluorescence staining was performed using γ H2AX antibody, as previously reported (Glaros *et al.*, 2012). Results of immunofluorescence staining and western blotting showed that YM155 effectively stimulated the expression of γ H2AX at nanomolar (nM)
10 concentrations in YM155-sensitive Mahlavu and HLE cells (Figure 5H and 5I) but not in YM155 resistant HuH7 and HepG2 cells (Figure 6B).

To further clarify the molecular mechanisms involved in the induction of cell death by YM155 towards sensitive HCC cells, microarray analysis was next performed to identify differentially expressed genes when Mahlavu cells were
15 treated with 10ng/ml YM155, 50nM survivin-specific siBIRC5 or the siRNA scramble control (Sigma). The knockdown effect of BIRC5 by siRNA was validated by qRT-PCR (Figure 7A). The cell viability assay showed that silencing BIRC5 decreased the sensitive Mahlavu cells to YM155 (Figure 7B). Bioinformatics analysis of the microarray data generated and coupled with the
20 Ingenuity Pathway Analysis (IPA) demonstrated that many cell cycle-, apoptosis- and DNA damage-related pathways were modulated (Figure 7C and 7D). Consistent with this observation, several cell cycle G2/M DNA damage

checkpoint regulatory-related genes including CCNB1, CKS2, WEE1 and API5 were found to be down-regulated while the growth arrest and DNA-damage-inducible protein GADD45 alpha (GADD45A) was up-regulated following treatment with either YM155 or siBIRC5 and tested by real time RT-qPCR (Figure 7E), suggesting YM155 induced cell death in the sensitive HCC cells may through survivin mediated apoptosis, cell cycle and G2/M DNA damage checkpoint regulation.

YM155 is a promising therapeutic agent targeting HCC with high survivin expression in pre-clinical evaluation using an orthotopic HCC xenograft mouse model

The pGL3 luciferase reporter vector was stably transfected into the Mahlavu and HuH7 cells. Then, the orthotopic mouse liver tumor xenograft models using Mahlavu and HuH7 luciferase expression cells was developed to test the in vivo efficacy of YM155 on human HCC tumor cells transplanted into immunodeficient mice. Luciferase-expressing Mahlavu and HuH7 cells were orthotopically implanted into the liver of nude mice and these mice were treated with YM155, sorafenib or saline for a total of 7 weeks. Sorafenib was administered at levels effective on multiple tumor xenografts (p.o., 30mg/kg, daily). YM155 (10mg/kg) was administered as a 7-day continuous infusion via intraperitoneal injections followed by observation for 7 days in 14-day treatment cycles. Tumor growth was measured every week by bioluminescence imaging using the Xenogen IVIS Lumina system once a week (Xenogen Corporation,

Hopkinton, MA). The results showed that YM155 markedly suppressed the growth of the Mahlavu cells and the therapeutic effect observed for YM155 was statistically better than sorafenib (Figures. 8A and 8B). In comparison, the observed therapeutic effect of YM155 on HuH7 was only marginal and sorafenib was more effective at suppressing the tumorigenesis of HuH7 cells (Figures 8C and 8D). The TUNEL staining result indicated that YM155 treatment induces significant much more tumor cell apoptosis than sorafenib treatment in tumors from Mahlavu cells, while the sorafenib treatment induce more tumor cell apoptosis than YM155 treatment in HuH7 cells (Figures. 8E and 8F). This may explain that the antitumor effect of YM155 was significantly better than sorafenib in Mahlavu cells, while sorafenib was more effective than YM155 at suppressing the tumor growth of HuH7 cells.

Several patient-derived HCC tumor xenograft models have been established . The orthotopic growth of three independent HCC patient-derived xenografts was examined for the expression and phosphorylation of survivin by western blotting. One of the patient-derived xenografts, tentatively designated P3, expressed a high level of survivin expression and phosphorylation (Figure 9A). Therefore, the P3 patient-derived xenograft was selected to test the therapeutic effect of YM155. It was observed that while the growth of P3 was moderately inhibited by sorafenib, the antitumor effect of YM155 was significantly better than that of sorafenib (Figures 9B-9D). Immunohistochemical analysis of tumor tissue samples from each treatment group indicated that the expression of

survivin was significantly inhibited in YM155 treated group. The TUNEL staining result indicated that YM155 treatment induces significant increasing of tumor cell apoptosis, while the sorafenib treated tumor induced fewer tumor cell apoptosis (Figure 9D). This may explain that the antitumor effect of YM155 was significantly better than that of sorafenib. These data suggest that YM155 is a promising therapeutic agent for targeting HCC cells with high survivin expression and phosphorylation.

Discussion

While chemotherapy is one of the standard methods of treatment for many human cancers, systemic chemotherapy has been generally ineffective in HCC. HCC has been shown to be chemoresistant to the most common chemotherapies (EASL and EORTC., 2012). Currently, sorafenib is the only molecular inhibitor that has been approved by the FDA in year 2007 as a first-line treatment for advanced HCC, but the treatment only increased progression-free survival by a paltry three months compared to placebo (Llovet *et al.*, 2008; Cheng *et al.*, 2009). Although new drugs such as sunitinib, erlotinib, linifanib, and brivanib are being developed to target HCC, they are not effective in all patients. The recent trial on mTOR inhibitor, Everolimus, also did not improve overall survival in patients with advanced HCC whose disease progressed during or after receiving sorafenib or who were intolerant of sorafenib (Zhu *et al.*, 2014).

Survivin (BIRC5) is a member of the IAPs family that is overexpressed in most human cancers and is not expressed in normal tissues. YM155, “survivin suppressant”, has been the subject of several phase I and phase II clinical trials targeting diffuse large cell lymphoma, prostate cancer, melanoma, and NSCLC, where only modest activity was noted (Holmes 2012). In the present study, it was demonstrated that the expression and phosphorylation of survivin was highly heterogeneous in HCC. This was further validated by RT-qPCR and IHC studies with independent HCC samples. Furthermore, we observed that the YM155-sensitive cells (Mahlavu and HLE) showed significantly high expression and phosphorylation of survivin while the YM155-resistant HCC cells (HuH7 and HepG2) gave relatively low survivin expression and phosphorylation. Additionally, YM155 inhibited survivin expression and anchorage-independent cell growth in sensitive liver cancer cells. Mechanistically, YM155 is highly cytotoxic *in vitro* against sensitive HCC cells and induces cell death, cell cycle arrest, apoptosis and DNA damage. Importantly, the observed therapeutic efficacy of YM155 against high survivin-expressing cells was shown to be significantly better than sorafenib when evaluated in preclinical animal studies.

Recently, it was reported that the solute carrier SLC35F2 enables YM155-mediated DNA damage toxicity by using a genome-wide insertional mutagenesis approach in the near-haploid human cell line KBM7. They found that SLC35F2 expression and YM155 sensitivity correlated across a panel of cancer cell lines, and targeted genome editing verified SLC35F2 as the main

determinant of YM155-mediated DNA damage toxicity *in vitro* and *in vivo* (Winter *et al.*, 2014). However, when the expression of SLC35F2 in HCC clinical samples dataset from this study and the reported HCC dataset of Roessler *et al.*, (2012) was examined, the expression difference of SLC35F2 in tumor and non-tumor samples of HCC was not observed (Figure 10A and 10B). The heterogeneity expression of SLC35F2 in HCC tumor samples was also not observed, which suggested that SLC35F2 could not be the main determinant to explain the remarkable heterogeneity sensitivity of HCC cells to YM155. The current data suggested that the protein expression and phosphorylation of survivin may guide the application of YM155 treatment for HCC in the design of the clinical trial. Therefore, it is useful to integration different prediction for future clinical application of YM155.

Several clinical studies of YM155 against human lung cancer, melanoma, lymphoma and prostate cancer have been completed. As a single agent, YM155 only produced modest activity in patients with refractory, advanced NSCLC (Giaccone *et al.*, 2009). The combination of YM155 with carboplatin and paclitaxel gave a favourable safety profile in advanced NSCLC but failed to produce an improvement in the response rate (Kelly *et al.*, 2013). The present data strongly suggest the sub-classification of HCC patients according to their survivin expression and phosphorylation to enhance the therapeutic efficacy of YM155. This was clearly demonstrated by the greater than 100-fold sensitivity to YM155 for the high survivin-expressing cells Mahlavu and HLE compared to

the low survivin-expressing cells, indicating that survivin-expression and phosphorylation can be one of a potential molecular companion biomarker for selecting YM155-sensitive HCC patients to achieve the delivery of precision medicine for cancer patients and could justify human studies to demonstrate dose and effectiveness (Schilsky 2014). This study indicated that survivin is a potential clinically actionable therapeutic target in HCC. We firstly demonstrated the preclinical evidence of antitumour activity of YM155 and evidence of target inhibition in model systems of HCC patients with high survivin expression and phosphorylation.

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Any listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that any such document is part of the state of the art or is common general knowledge.

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Claims

1. A method for profiling a liver sample from a hepatocellular carcinoma subject comprising:
 - 5 (i) determining survivin gene expression level and/or survivin protein phosphorylation in the sample;
 - (ii) comparing the survivin gene expression level and/or survivin protein phosphorylation in the sample with survivin gene expression level(s) and/or survivin protein phosphorylation, respectively from at least one control; and
 - 10 (iii) classifying the sample as either having higher, lower or equal gene expression levels compared to the control.
2. A method for treating HCC comprising:
 - (i) determining survivin gene expression level and/or survivin protein phosphorylation in a liver sample from a HCC subject;
 - 15 (ii) comparing the survivin gene expression level and/or survivin protein phosphorylation in the liver sample with survivin gene expression level and/or survivin protein phosphorylation, respectively from at least one control; and
 - (iii) administering at least one survivin inhibitor to a subject classified
20 as having a higher survivin gene expression level compared to the control.
3. The method according to claim 1 or 2, wherein the liver sample is taken from a liver neoplasm.

4. The method according to any one of claims 1 to 3, wherein the control comprises at least one normal liver tissue sample.
5. The method according to claim 4 as dependent on claim 1 or both claims 1 and 3, further comprising identifying a subject with higher
5 survivin gene expression level and/or survivin protein phosphorylation compared to the control as likely to have a higher recurrence of hepatocellular carcinoma.
6. A method for monitoring a HCC subject comprising determining survivin gene expression levels and/or survivin protein phosphorylation of
10 (a) at least one liver sample taken from the subject at a first time point before the subject has been administered at least one survivin inhibitor and (b) at least one liver sample separately taken from the subject taken
at various subsequent time points after the subject has been administered the at least one survivin inhibitor, wherein:
15 (i) lower survivin gene expression level and/or survivin protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding positively to the survivin inhibitor;
(ii) higher survivin gene expression level and/or survivin protein
20 phosphorylation at a subsequent time point compared to the first time point and/or a preceding subsequent time point, is indicative that the HCC subject is responding poorly to the survivin inhibitor; and
(iii) substantially the same survivin gene expression level and/or survivin
25 protein phosphorylation at a subsequent time point compared to the first time point and/or a preceding time point is indicative that the HCC subject is not responding to the survivin inhibitor.

7. The method according to any one of the preceding claims, wherein determining survivin gene expression level(s) is at the transcription and/or translation level(s).
- 5 8. The method according to claim 7, wherein determining the survivin gene expression level(s) at the transcription level comprises northern blot analysis, microarray analysis and/or reverse-transcription PCR.
9. The method according to claim 7, wherein determining the survivin gene expression level(s) at the translation level and/or survivin protein phosphorylation comprises Western blot, immunohistochemistry (IHC) and/or enzyme-linked immunosorbent analysis (ELISA) analysis.
- 10 10. The method according to any one of claim 1 and its dependent claims 3 to 5 and 7 to 9; and 6 and its dependent claims 7 to 9, wherein the method comprises an *in vitro* method.
11. A method for treating HCC in a subject comprising administering at least one survivin inhibitor to the subject.
- 15 12. The method according to any one of claim 2 and its dependent claims 3 to 4 and 7 to 10; and claims 6 and 11, wherein said at least one survivin inhibitor inhibits survivin gene expression, survivin protein activity and/or survivin protein phosphorylation.
- 20 13. The method according to claim 12, wherein said at least one survivin inhibitor inhibiting survivin gene expression comprises at least one RNA interfering agent targeting the survivin mRNA.
14. The method according to claim 13, wherein said at least one RNA interfering agent comprises at least one small interfering RNA (siRNA).

15. The method according to claim 12, wherein said at least one survivin inhibitor inhibits survivin protein activity.
16. The method according to claim 11, 12 or 15, wherein the survivin inhibitor comprises 1-(2-methoxyethyl)-2-methyl-3-(pyrazin-2-ylmethyl)-2H-benzo[f]benzimidazole-4,9-dione;bromide (YM155).
17. The method according to claim 11, 12 or 15, wherein the survivin inhibitor comprises at least one antibody and/or a functional fragment thereof.
18. The method according to claim 17, wherein the at least one antibody comprises a monoclonal antibody or comprises polyclonal antibodies.
19. A survivin inhibitor for use in treating HCC in a subject.
20. The survivin inhibitor for the use according to claim 19, wherein the survivin inhibitor inhibits survivin gene expression, survivin protein activity and/or survivin protein phosphorylation..
21. The survivin inhibitor for the use according to claim 20, wherein the survivin inhibitor inhibiting survivin gene expression comprises RNA interfering agent targeting survivin mRNA.
22. The survivin inhibitor for the use according to claim 21, wherein the RNA interfering agent comprises a small interfering RNA (siRNA).
23. The survivin inhibitor for the use according to claim 20, wherein said at least one survivin inhibitor inhibits survivin protein activity protein.
24. The survivin inhibitor for the use according to claim 19, 20 or 23, wherein the survivin inhibitor comprises YM155.

25. The survivin inhibitor for the use according to claim 19, 20 or 23, wherein the at least one survivin inhibitor comprises at least one antibody and/or a functional fragment thereof.
26. The survivin inhibitor for the use according to claim 25, wherein the at least one antibody comprises a monoclonal antibody or comprises polyclonal antibodies.
27. The survivin inhibitor for the use according to any one of claims 19 to 26, wherein a HCC sample from the subject has higher survivin gene expression levels compared to at least one normal liver tissue sample.
28. The survivin inhibitor for the use according to claim 27, wherein the subject is profiled according to the method of claim 1 and its dependent claims 3-5 and 7-10.
29. Use of a survivin inhibitor in the preparation of a medicament for treating HCC in a subject.
30. Use according to claim 29, wherein the survivin inhibitor inhibits the survivin gene expression, survivin protein activity and/or survivin protein phosphorylation.
31. Use according to claim 30, wherein the survivin inhibitor inhibiting survivin gene expression comprises RNA interfering agent targeting survivin mRNA.
32. Use according to claim 31, wherein the RNA interfering agent comprises a small interfering RNA (siRNA).
33. Use according to claim 29, wherein the survivin inhibitor inhibits activity of survivin protein activity.

34. Use according to claim 29, 30 or 33, wherein the survivin inhibitor comprises YM155.
35. Use according to claim 29, 30 or 33, wherein the survivin inhibitor comprises at least one antibody and/or a functional fragment thereof.
- 5 36. Use according to claim 35, wherein the at least one antibody comprises a monoclonal antibody or comprises polyclonal antibodies.
37. Use according to any one of claims 29 to 36, wherein a HCC sample from the subject has higher survivin gene expression level and/or survivin protein phosphorylation compared to at least one normal liver
10 tissue sample.
38. Use according to claim 37, wherein the subject is profiled according to the method of claim 1 and its dependent claims 3-5 and 7-10.

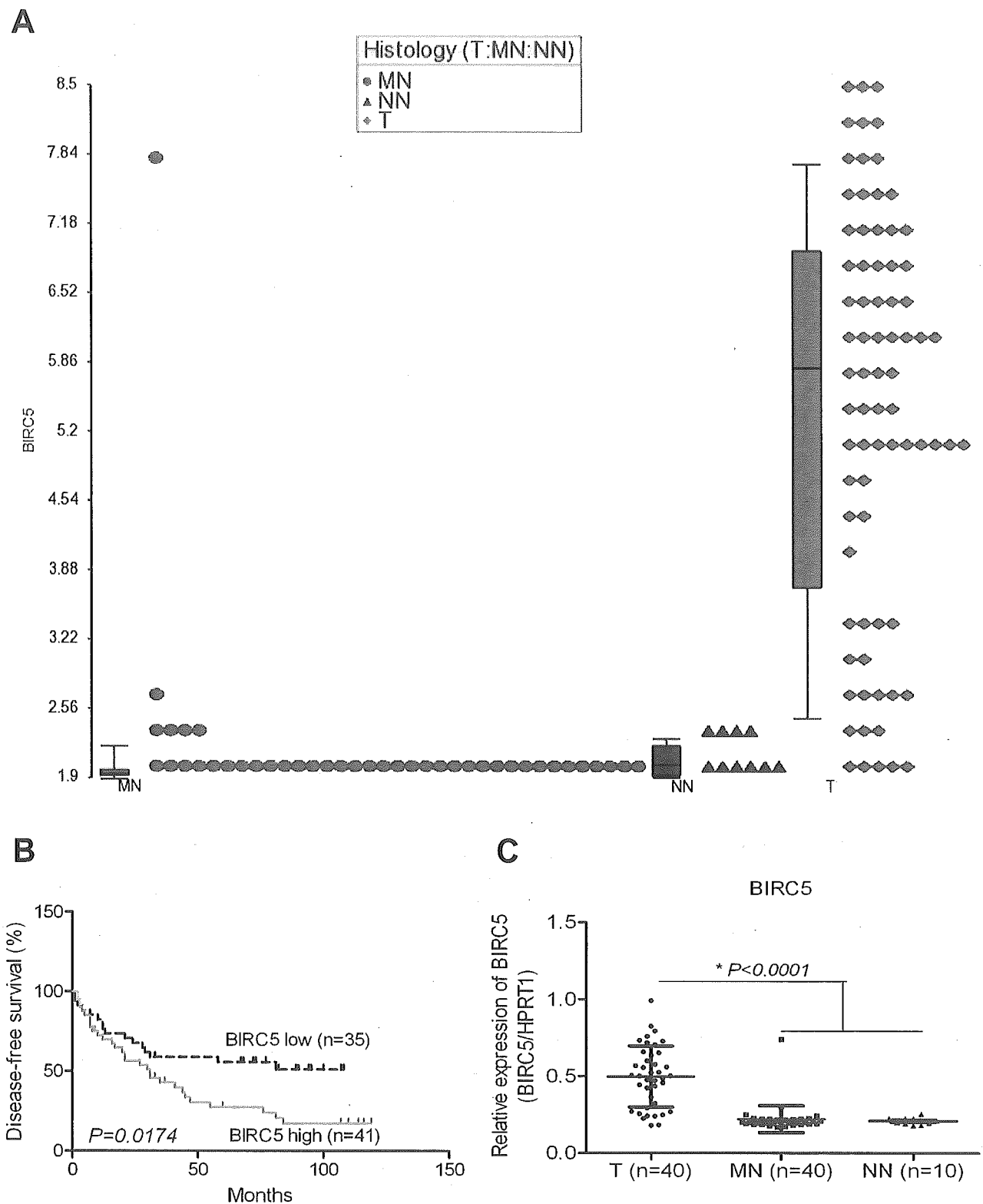
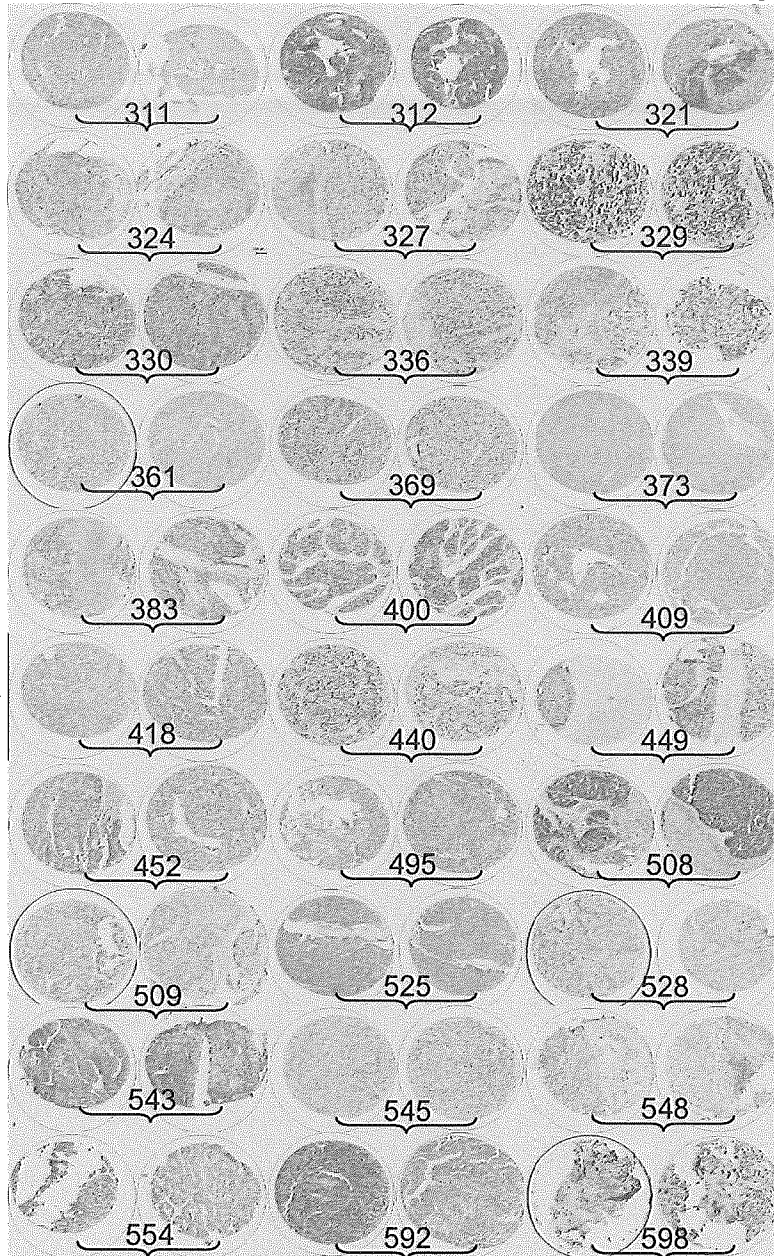
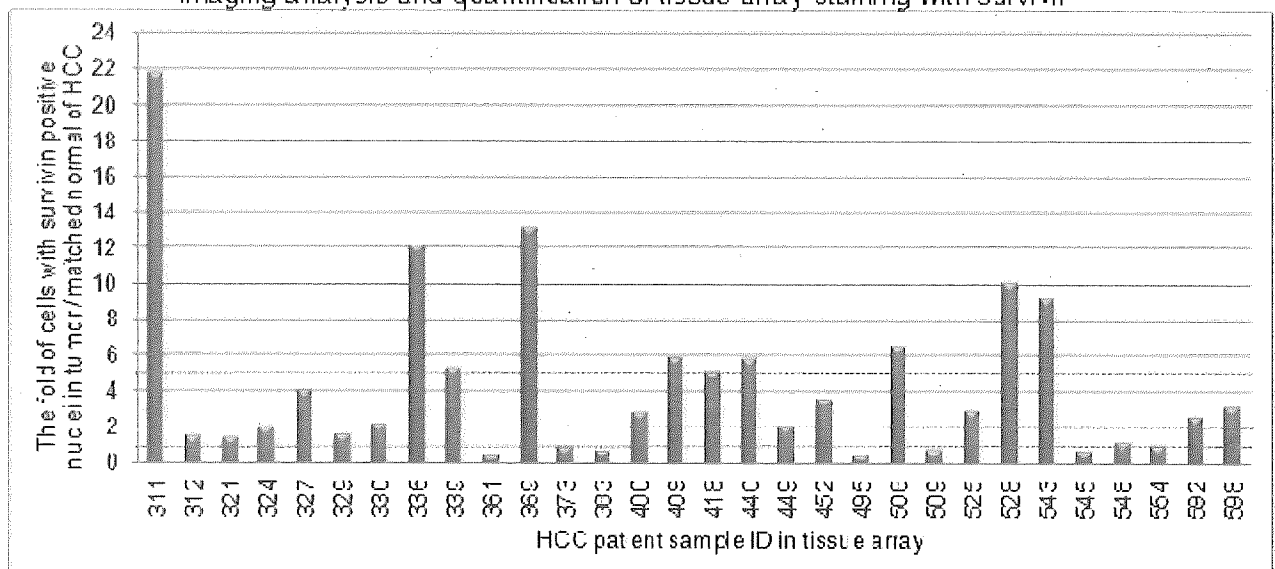


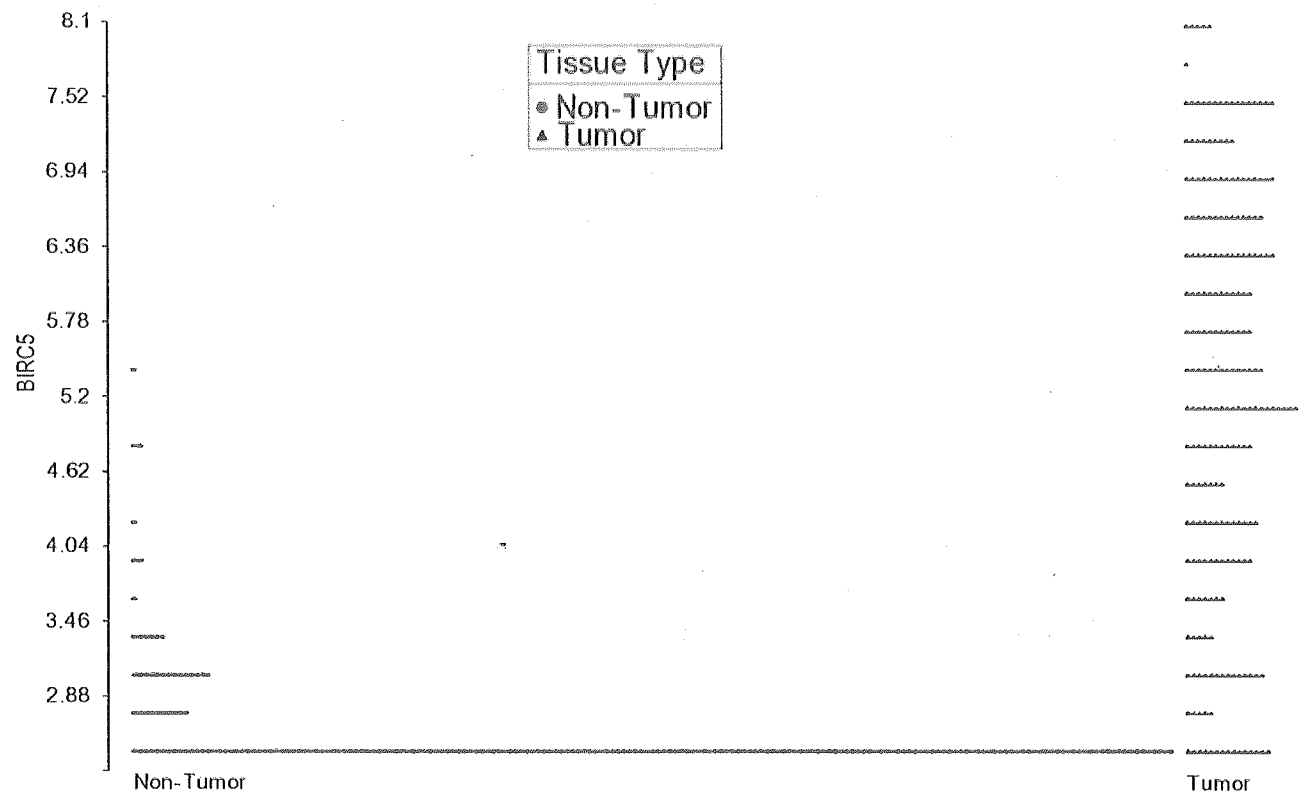
Figure 1

D**E**

Imaging analysis and quantification of tissue array staining with survivin

**Figure 1 (continued)**

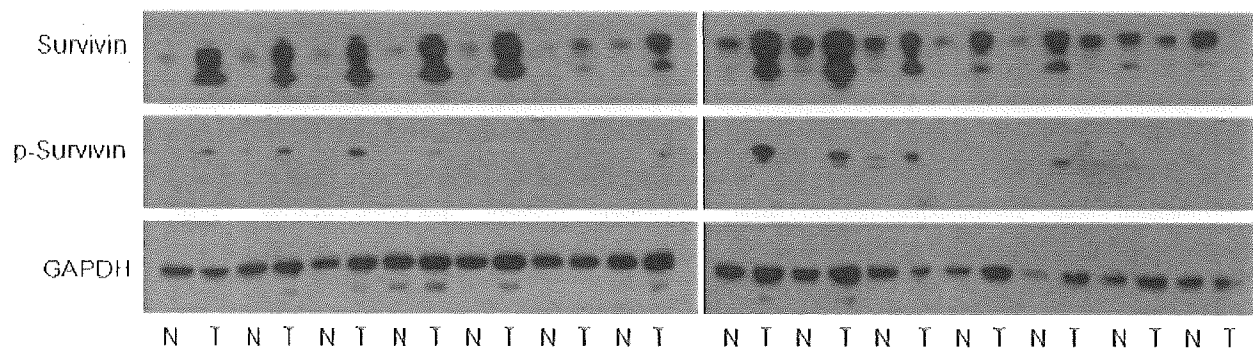
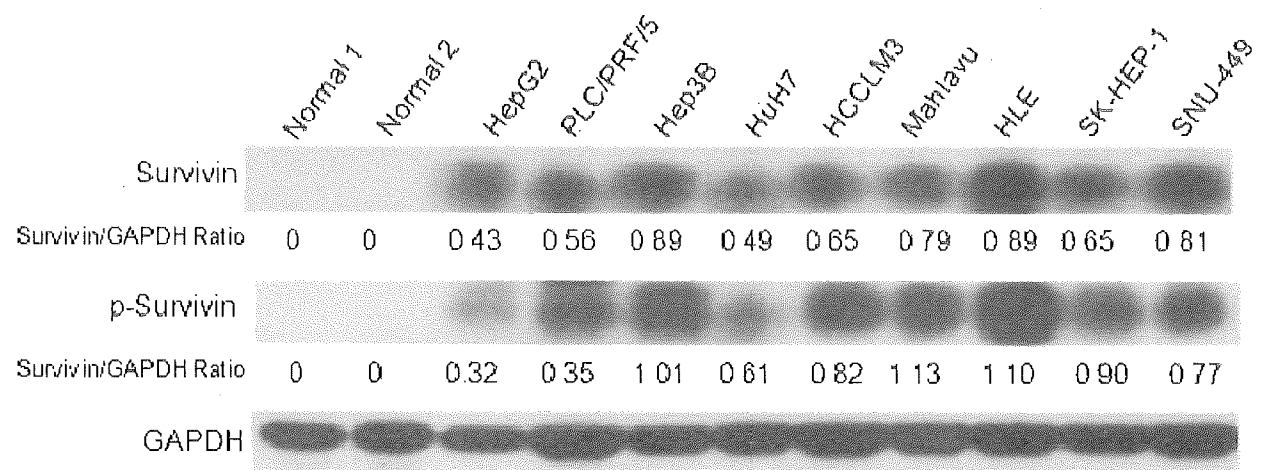
A The heterogeneous expression of BIRC5 in 445 cases of HCC tumors and non-tumors



B

TMA102														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	311				312				321				324	
B			327				329				330			
C	336				339				361				369	
D			373				383				400			
E	409				418				440				449	
F			452				495				508			
G	509				525				528				543	
H			545				548				554			
I	592				X	X	598				X	X	X	X

Figure 2

A**B****Figure 3**

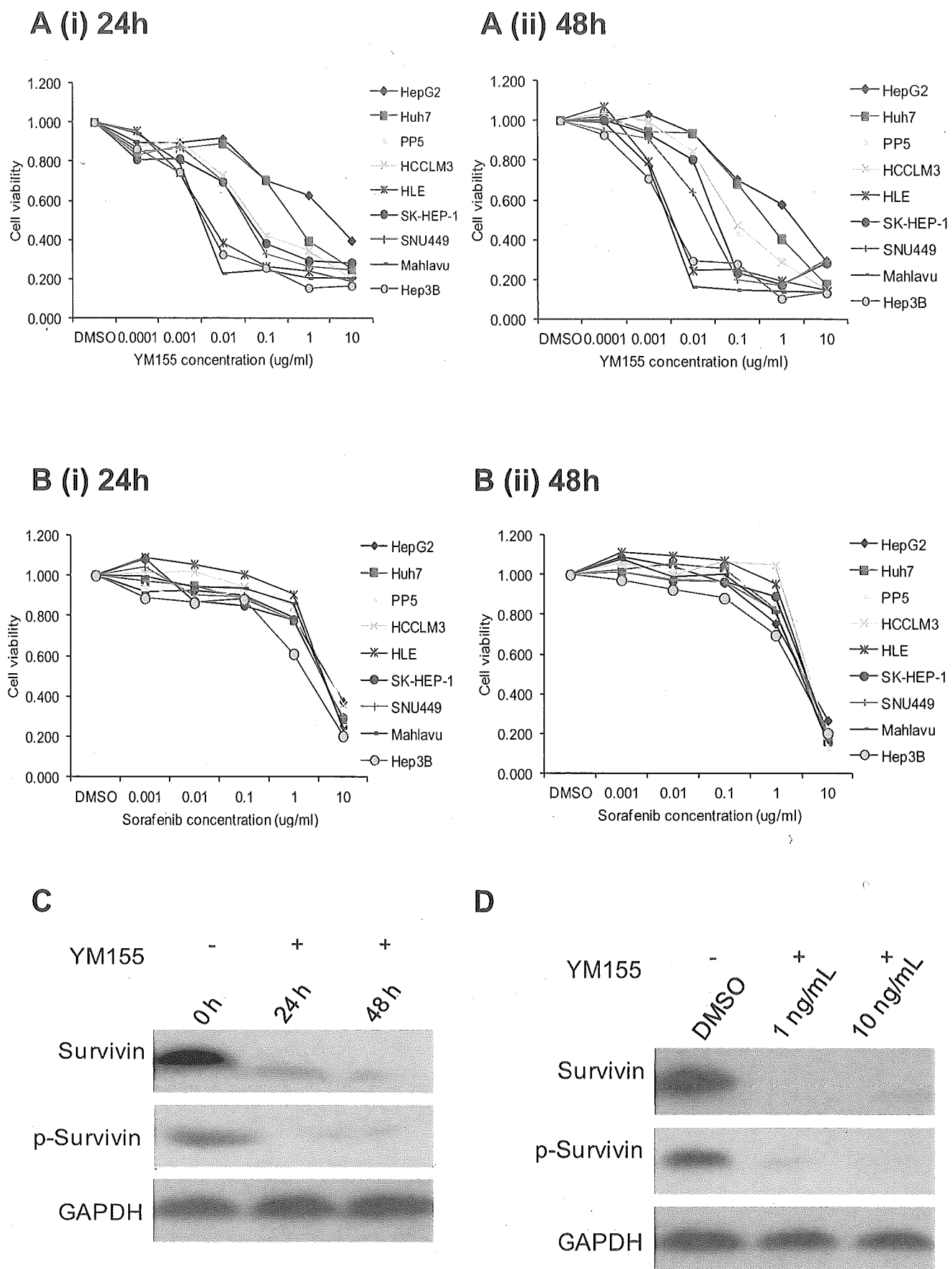
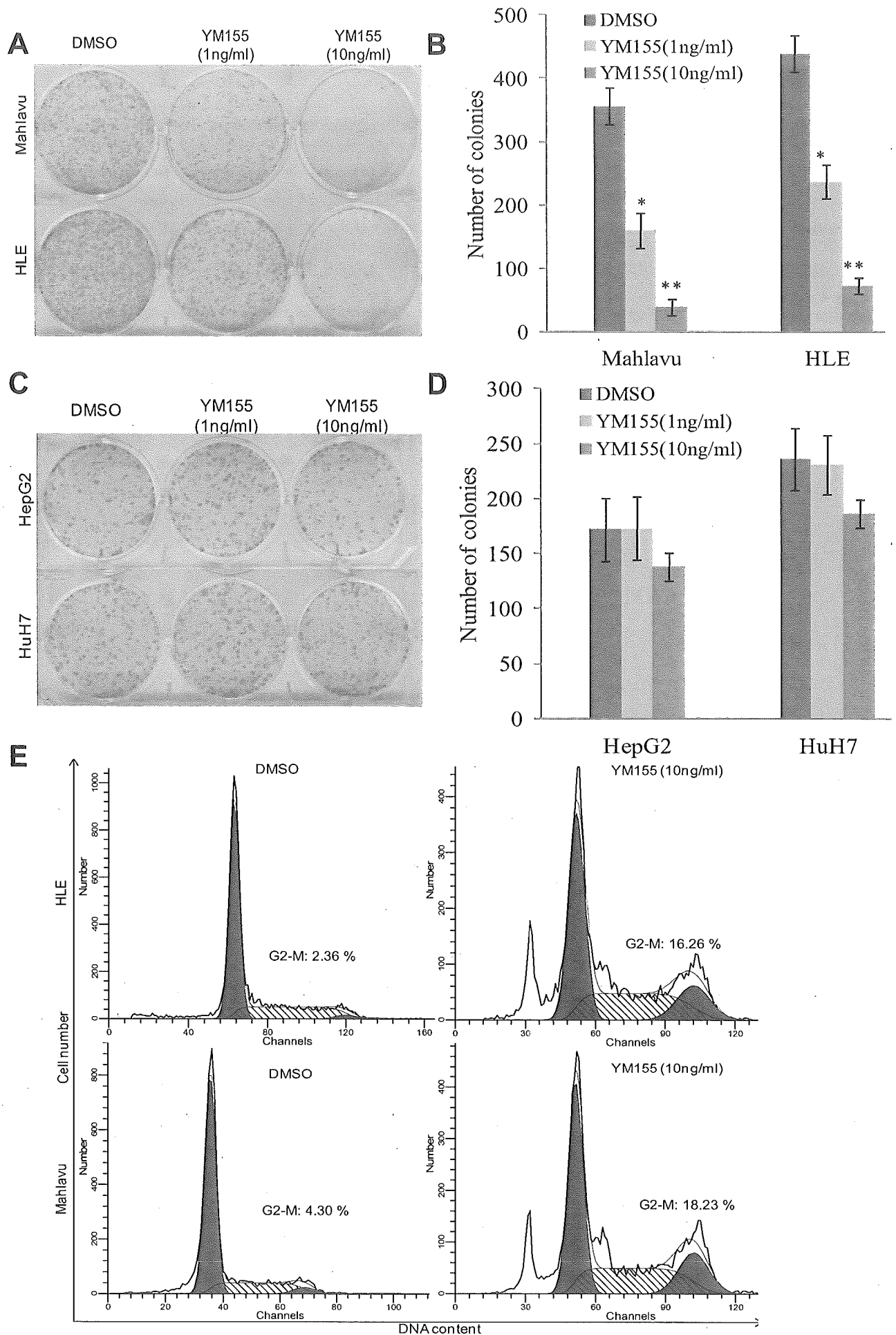
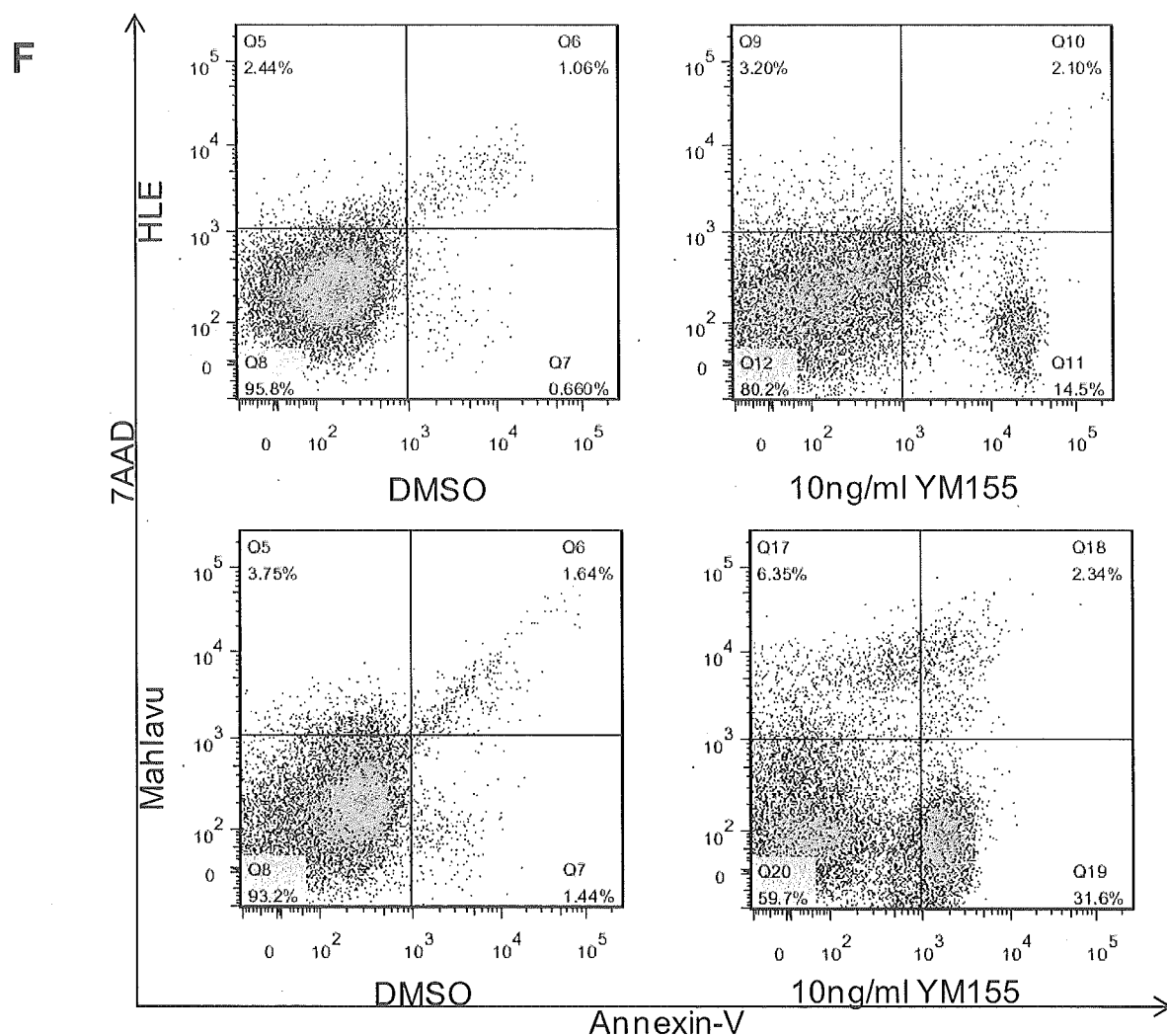


Figure 4

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G

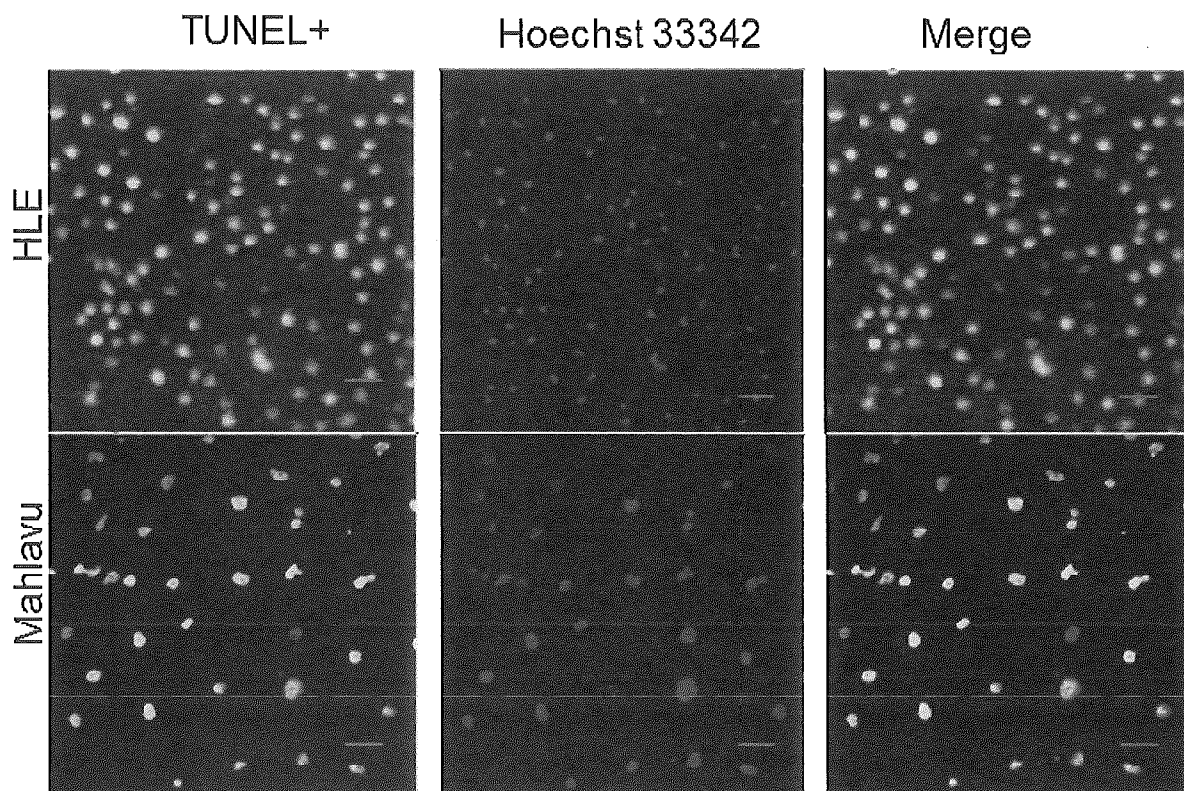
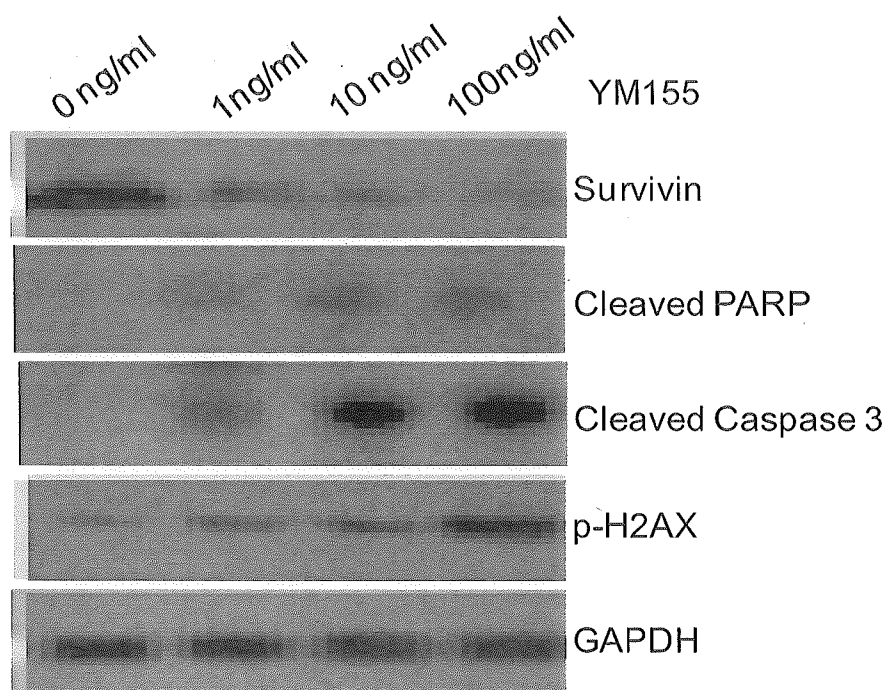
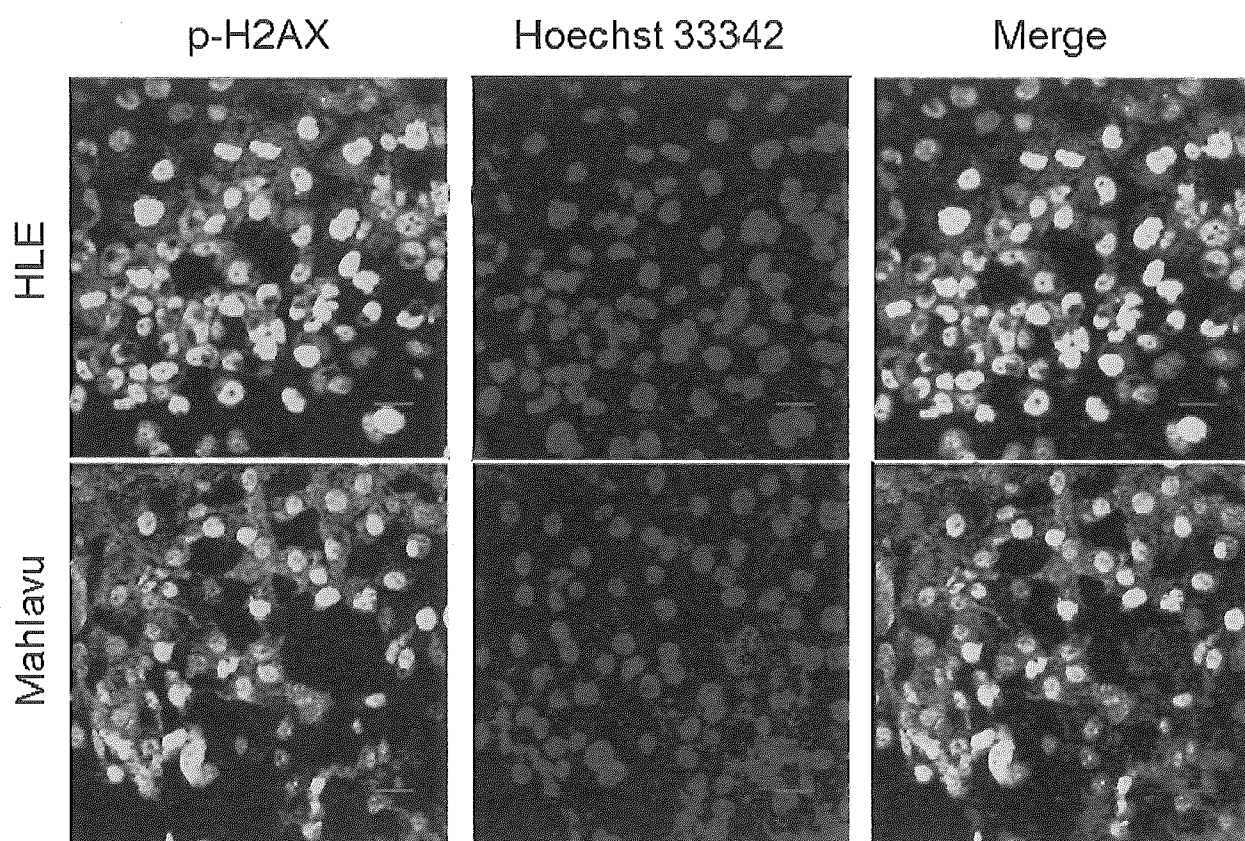
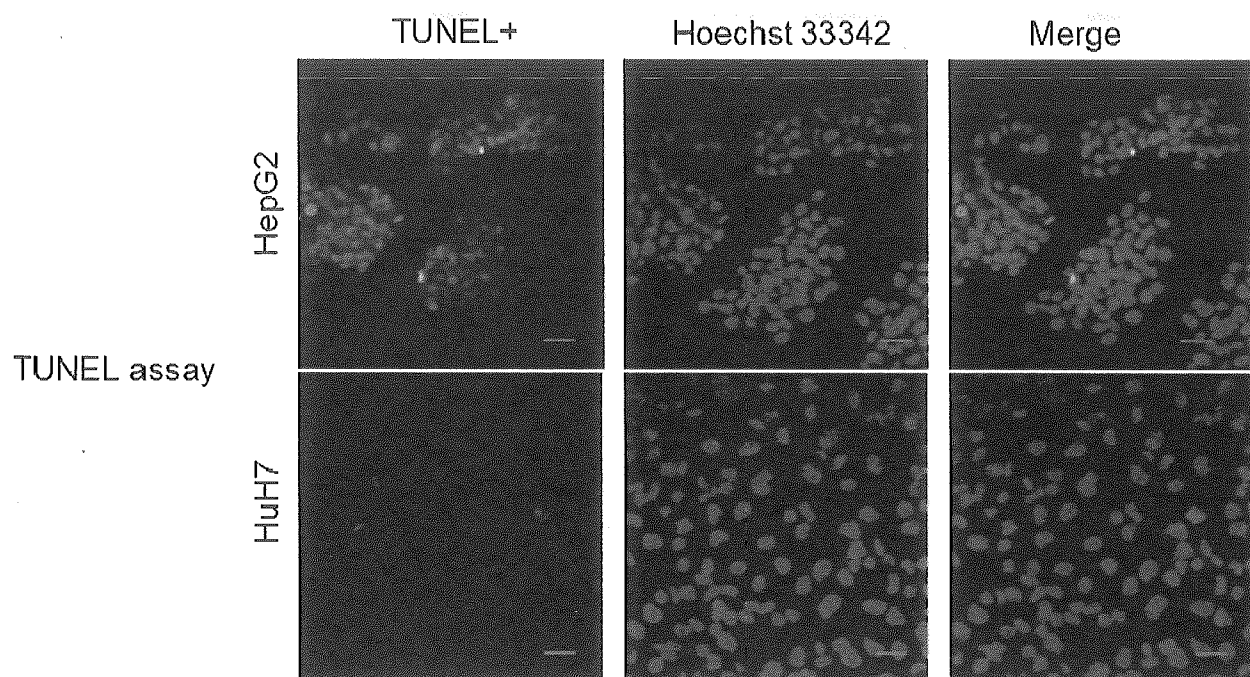
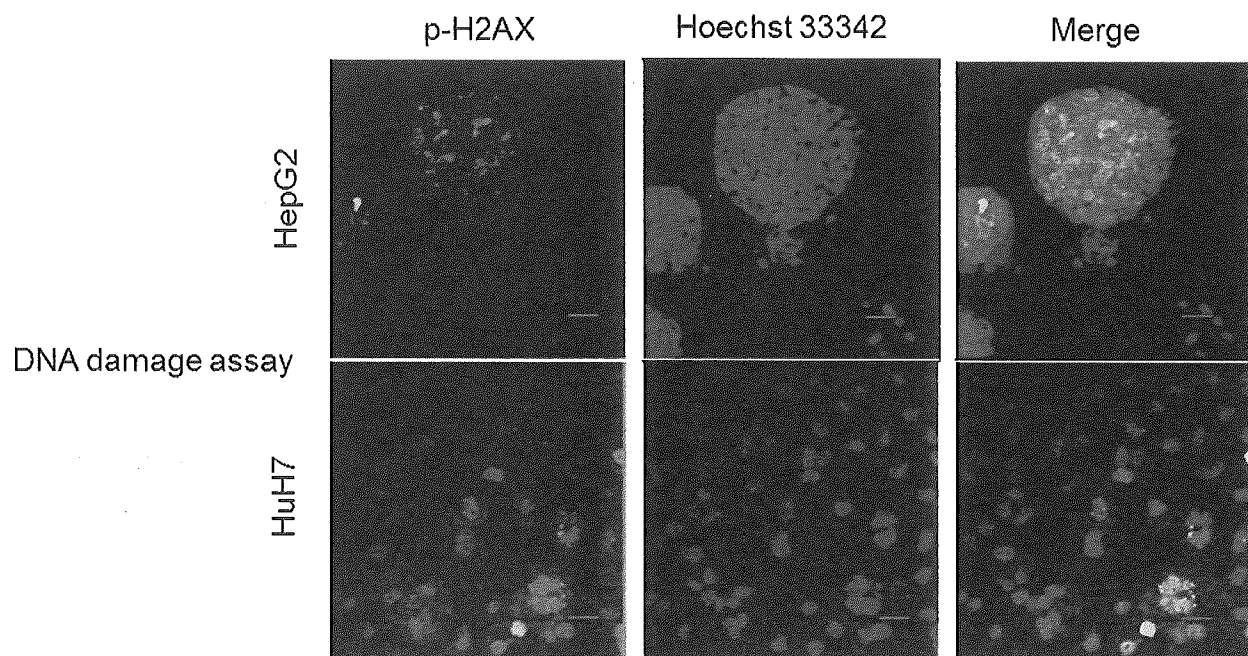


Figure 5 (continued)
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H**I****Figure 5 (continued)**

A**B****Figure 6**

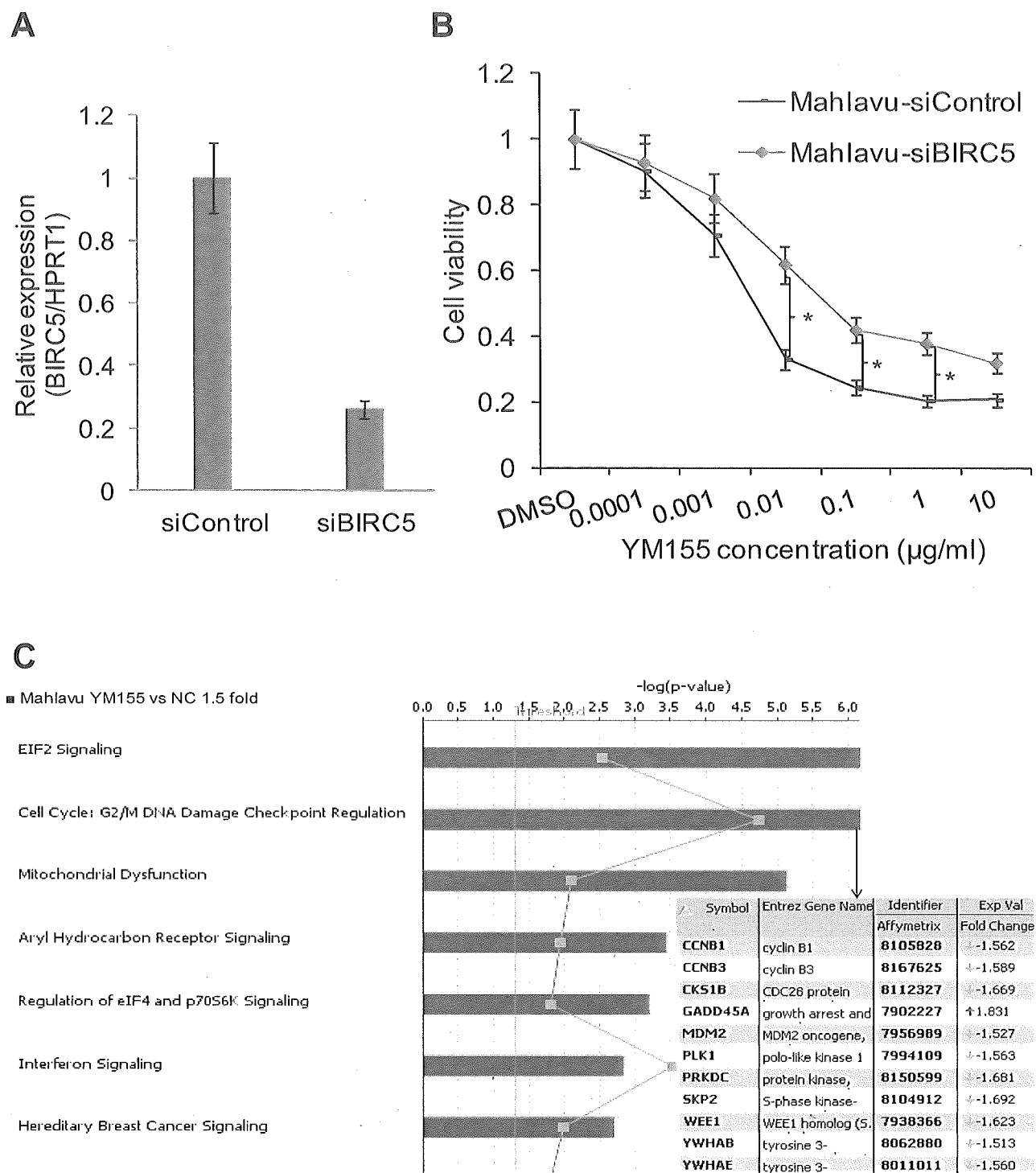
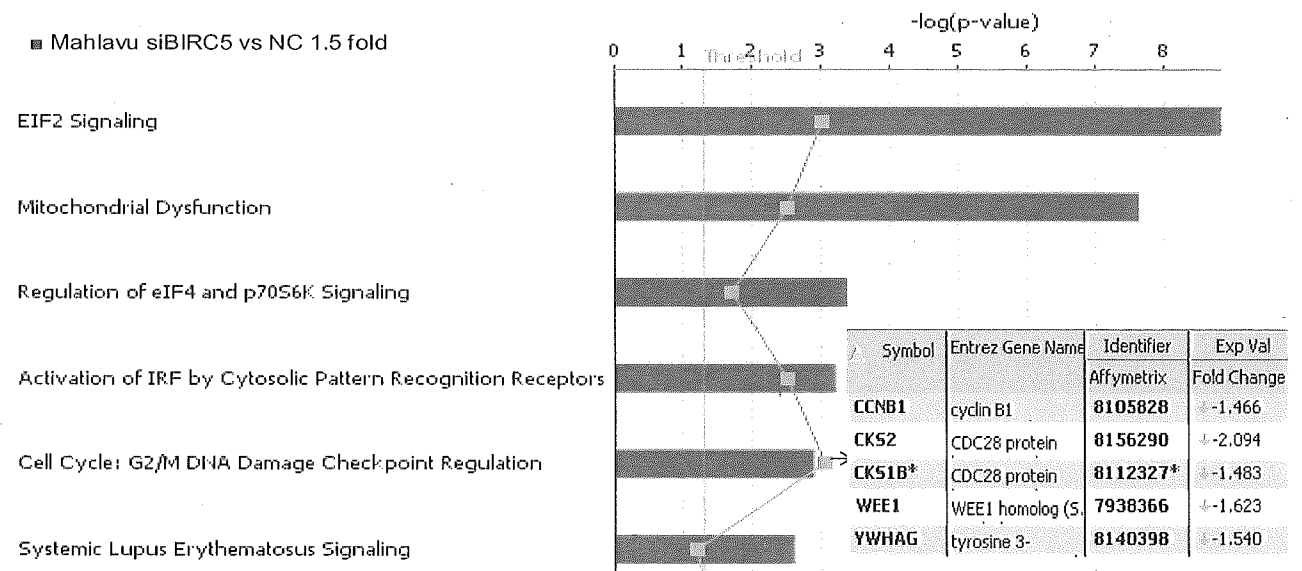
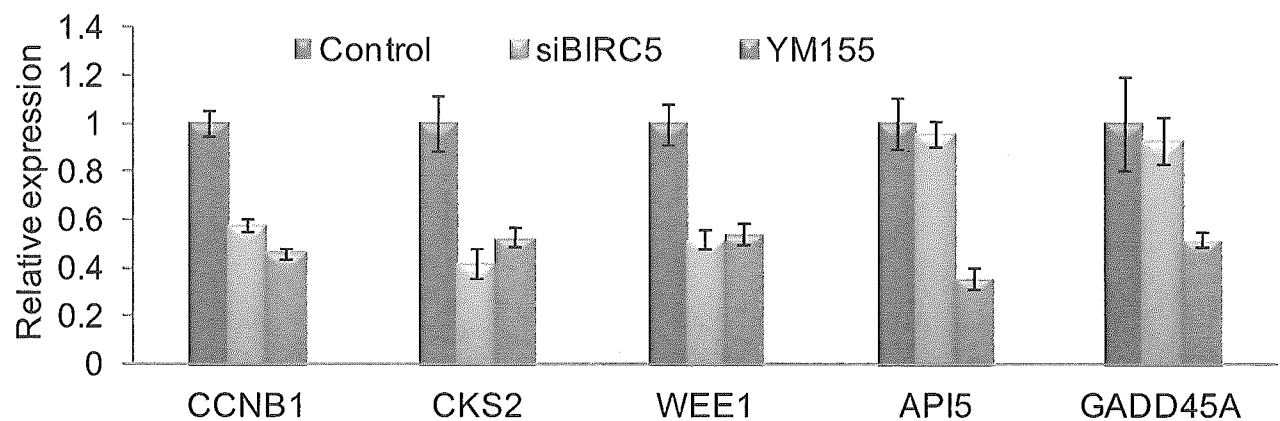
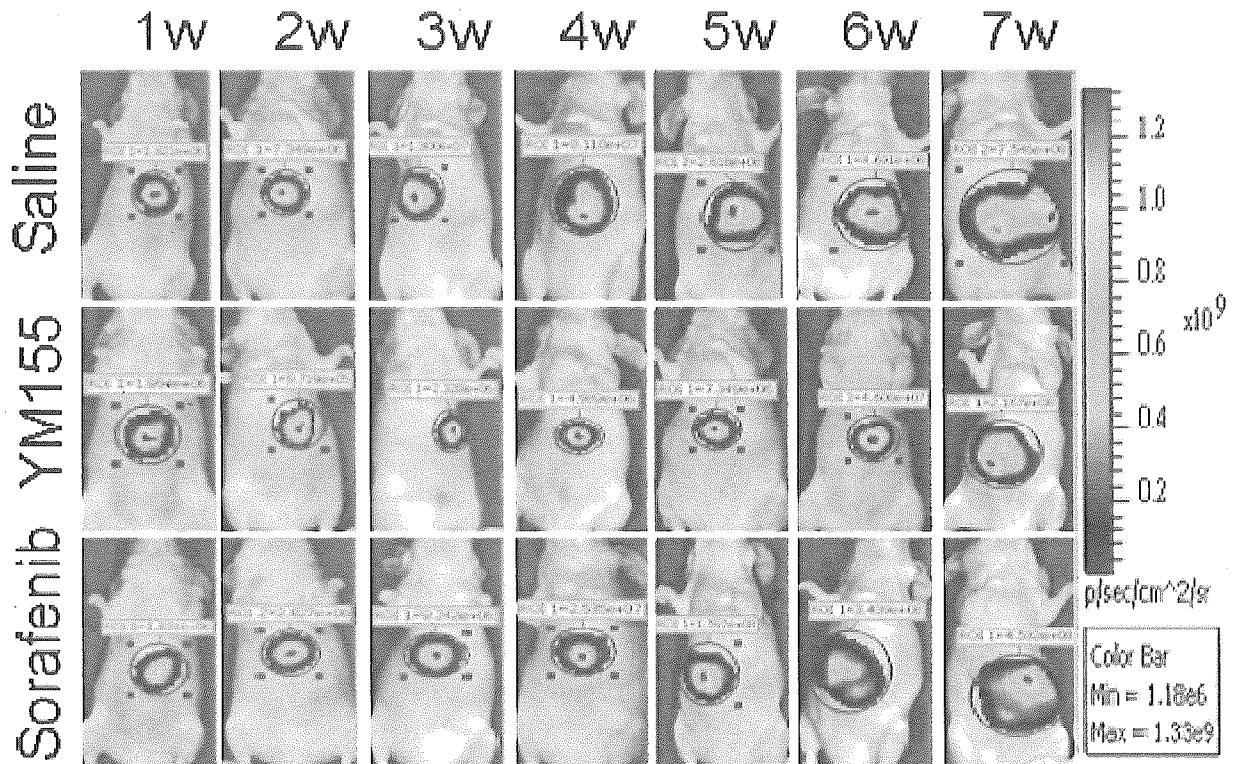
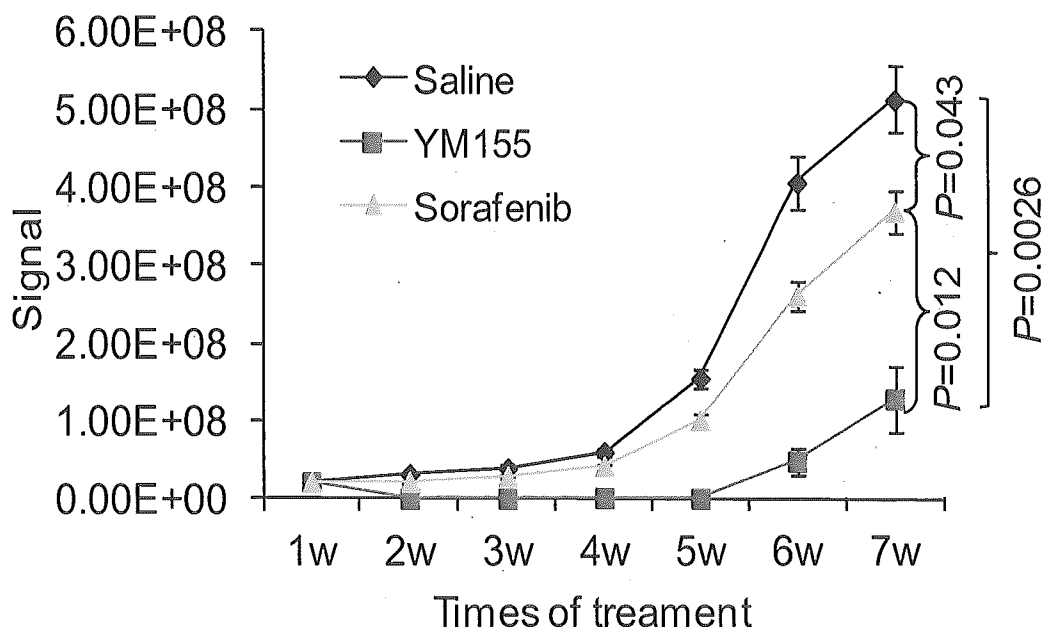


Figure 7

D**E****Figure 7 (continued)**

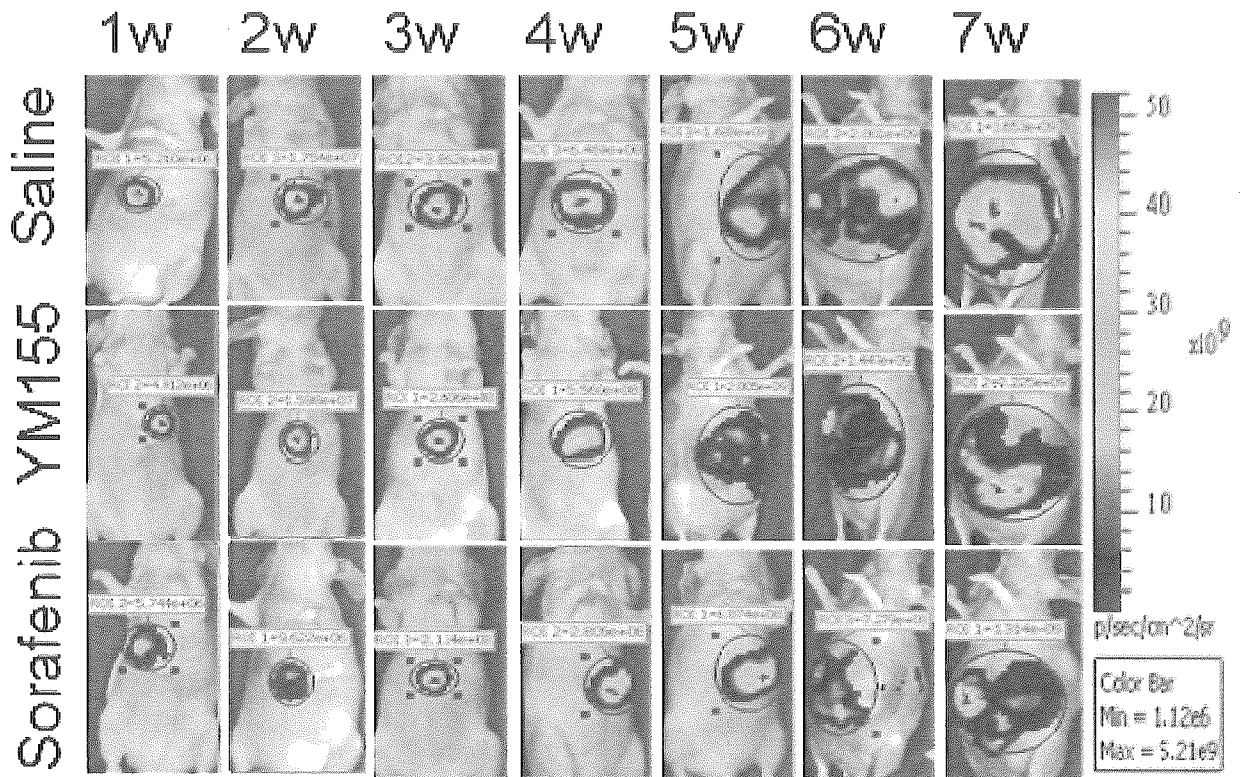
A**B**

Mahlavu-luciferase cells mice

**Figure 8**

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C



D

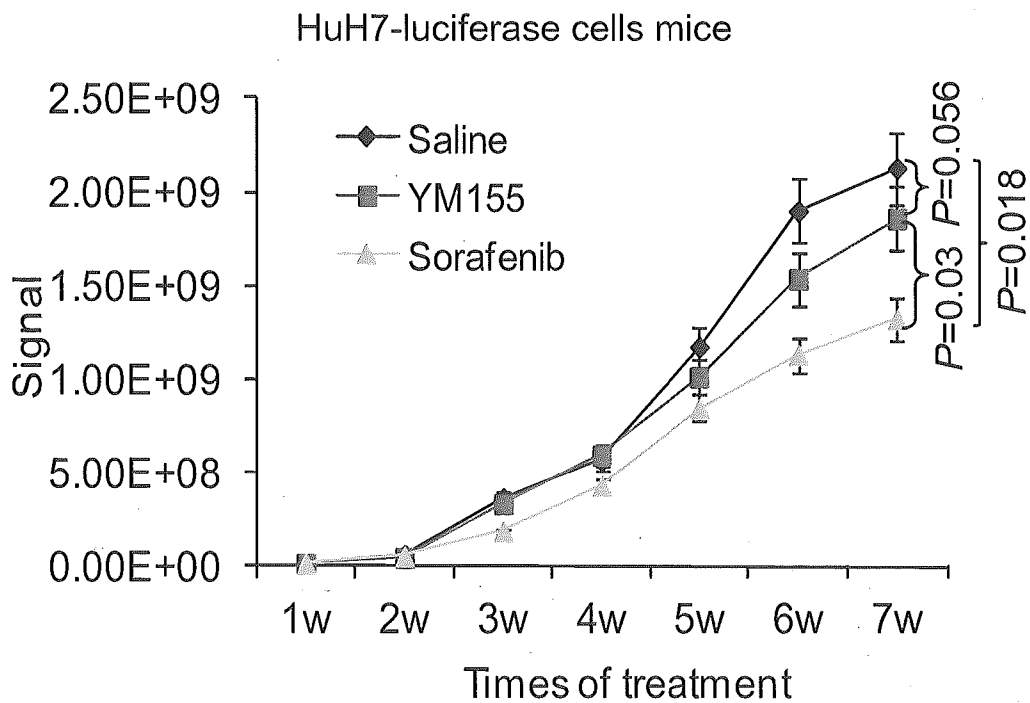
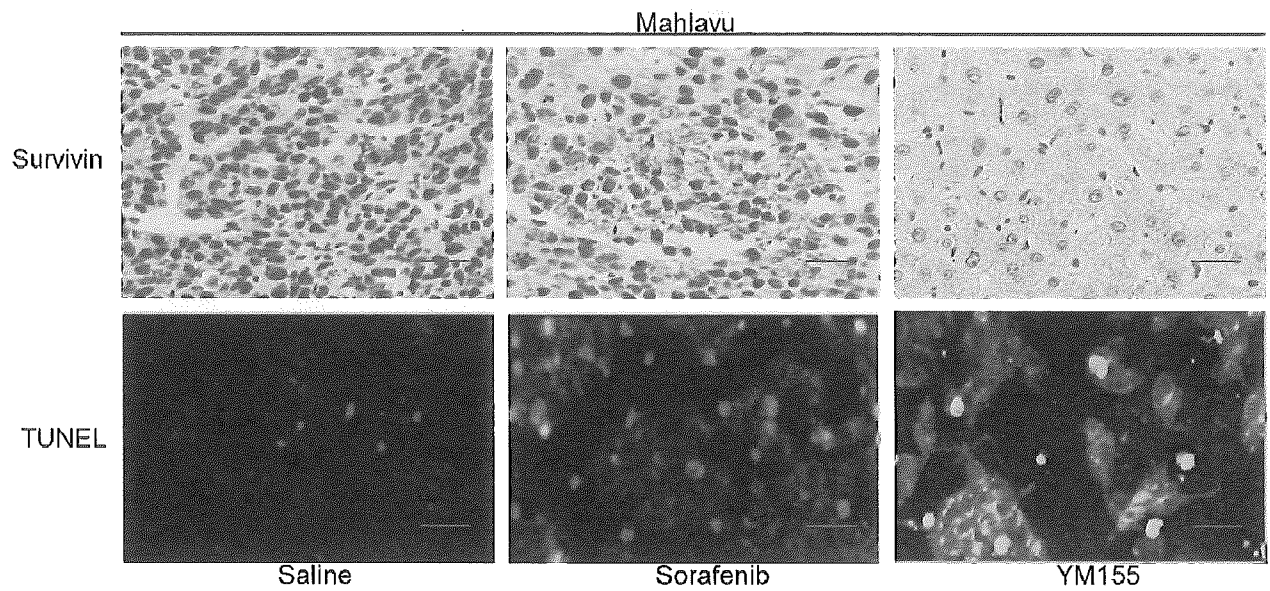
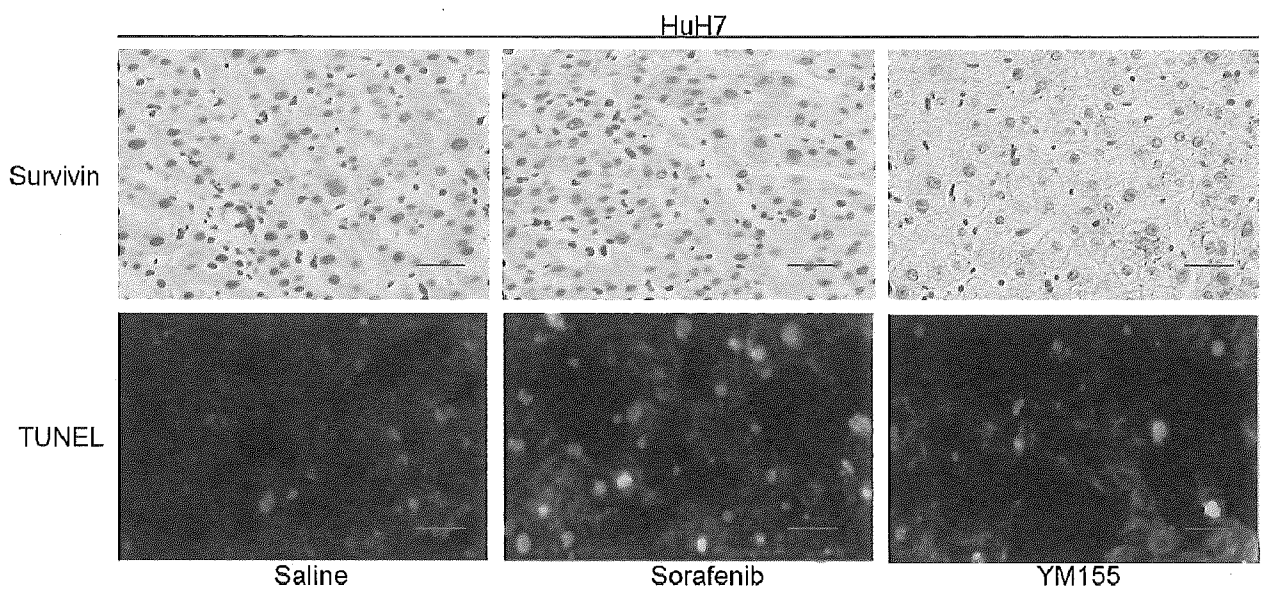


Figure 8 (continued)

E**F****Figure 8 (continued)**

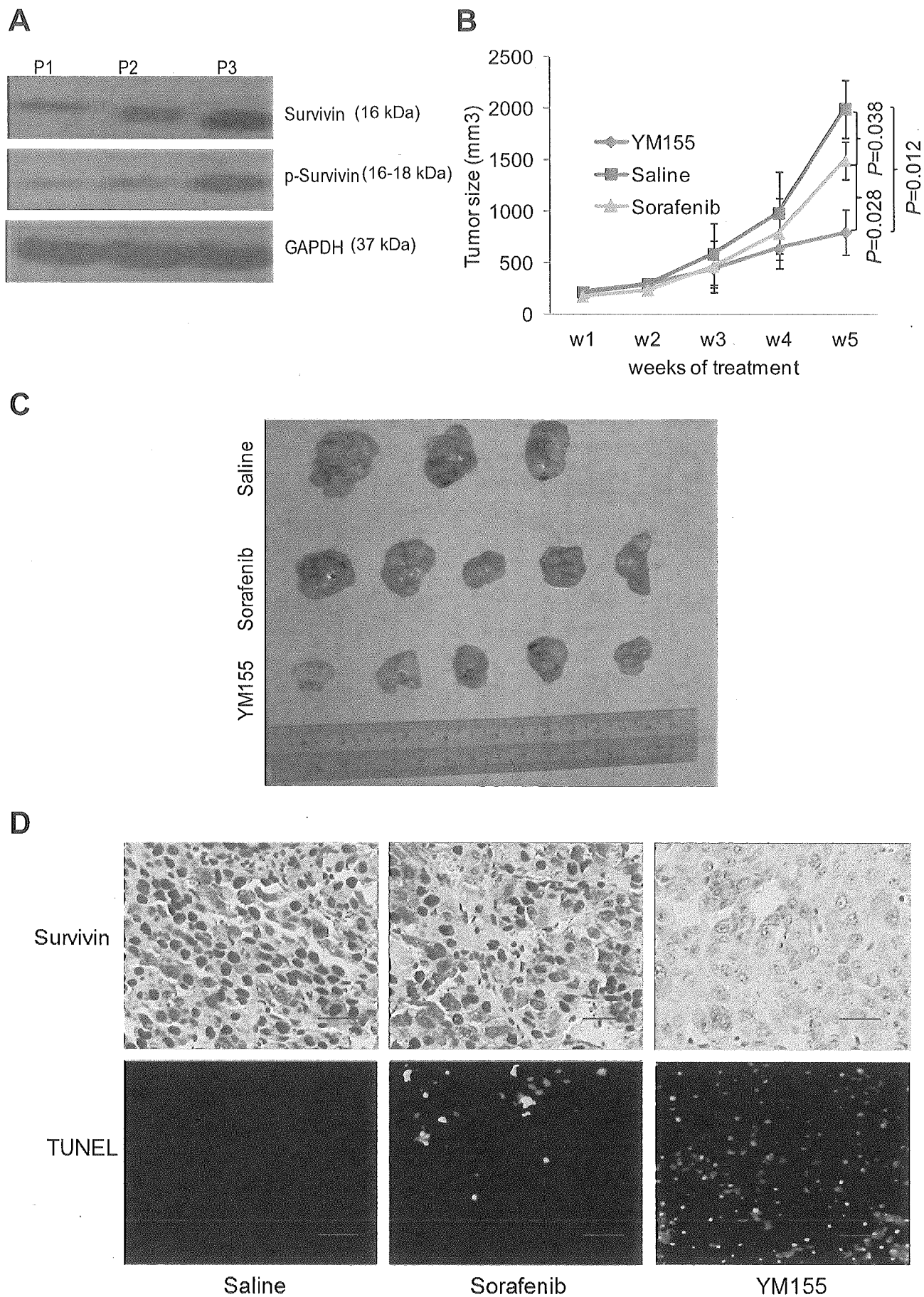
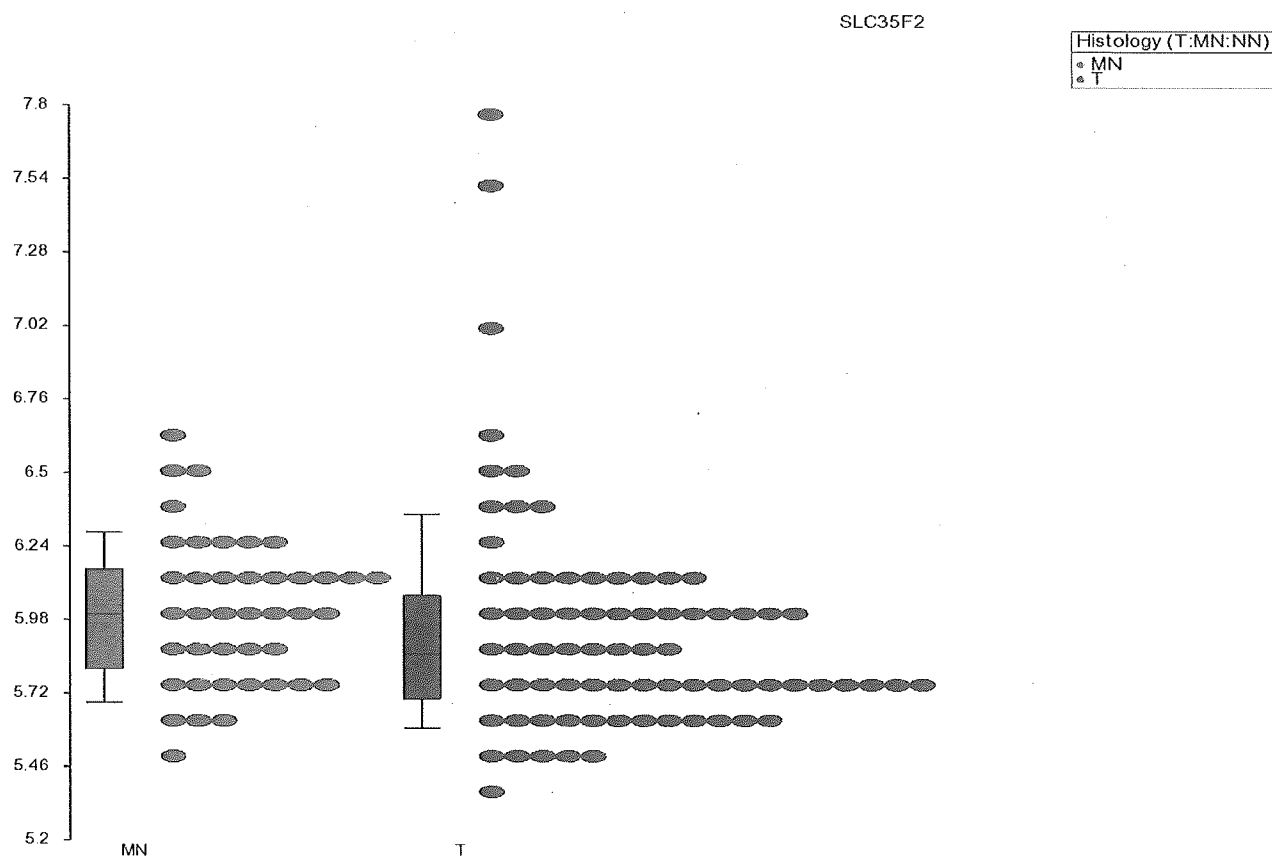


Figure 9

SUBSTITUTE SHEET (RULE 26)

A



B

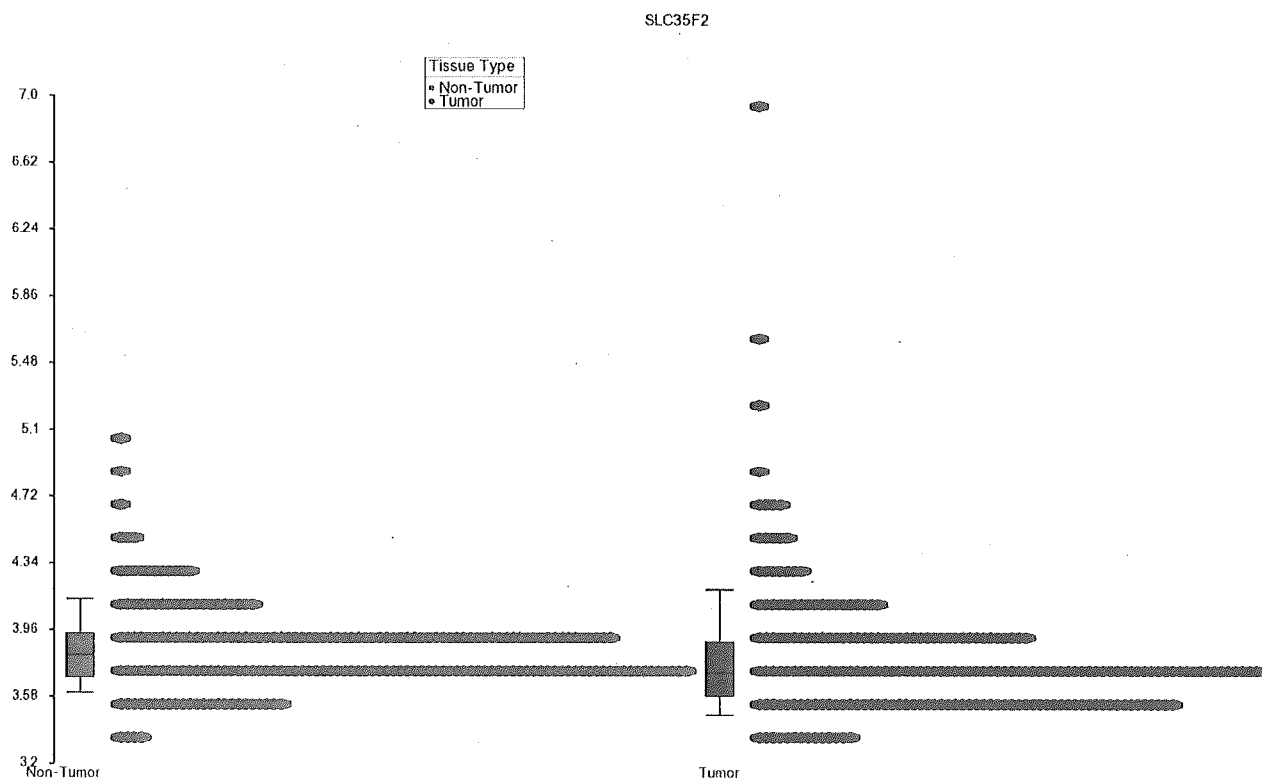


Figure 10
SUBSTITUTE SHEET (RULE 26)

A. CLASSIFICATION OF SUBJECT MATTER**A61K 31/7105 (2006.01) C12Q 1/68 (2006.01) G01N 33/574 (2006.01)**

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)

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)

Databases: TXPEA, TXPEB, TXPEC, TXPEE, TXPEF, TXPEI, TXPEP, TXPEPEA, TXPES, TXPUS01A, TXPUSE1A, TXPUSEA, TXPUSEB, TXPWOEA, WPIAP, EPODOC, MEDLINE, BIOSIS, HCAPLUS, EMBASE with KEYWORDS: hepatocellular carcinoma, liver cancer, survivin, BIRC5, inhibitor, siRNA, YM155, LY2181308 & like terms.

Databases: PAMS NOSE, INTESS, AusPat, The Lens, Google with keywords: Applicant / Inventor names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 7 September 2015	Date of mailing of the international search report 07 September 2015
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Catriona Bruce AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. + 61 2 6283 7940

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2015/050229
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/148198 A1 (DANA-FARBER CANCER INSTITUTE, INC.) 03 October 2013 paras [0006-0007]; Claims; paras [0060-0065]; paras [0079-0087]	11-36
X	HAN, S., et al., 'Polyamidoamine dendrimer liposome-mediated survivin antisense oligonucleotide inhibits hepatic cancer cell proliferation by inducing apoptosis', Tumor Biology, 2014 (epub 3 Feb 2014), vol. 35, pages 5013-5019 Abstract; Materials and Methods	11-36
X	US 2008/0038736 A1 (LLOVET et al.) 14 February 2008 Abstract; Claims; para [0038]; Figure 3	1, 3-5, 7-10
X	LIU, J. L., et al., 'Meta-analysis: prognostic value of survivin in patients with hepatocellular carcinoma', PLoS ONE, 2013, vol. 8, e83350 Abstract; page 3, left hand column, lines 13-14	1, 3-5, 7-10
X	XIA, H., et al., 'The over-expression of survivin enhances the chemotherapeutic efficacy of YM155 in human hepatocellular carcinoma', Oncotarget, 21 January 2015, vol. 6, pages 5990-6000 Abstract; Figure 2A; Figure 6A; Figure 3 C, D; page 5991, right hand column, line 8	1-10, 12-18, 37-38 (insofar as these claims relate to the option of determination of survivin protein phosphorylation)
P,X	XIA, H., et al., 'The over-expression of survivin enhances the chemotherapeutic efficacy of YM155 in human hepatocellular carcinoma', Oncotarget, 21 January 2015, vol. 6, pages 5990-6000 Abstract; Figure 2A; Figure 6A; Figure 3 C, D; page 5991, right hand column, line 8	1-10, 12-18, 37-38 (insofar as these claims relate to the option of determination of survivin expression levels only) and claims 11, 19-36

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/SG2015/050229	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2013/148198 A1	03 October 2013	WO 2013148198 A1	03 Oct 2013
		US 2015065518 A1	05 Mar 2015
US 2008/0038736 A1	14 February 2008	US 2008038736 A1	14 Feb 2008
		US 8030013 B2	04 Oct 2011
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			