High expression of HOXA13 correlates with poorly differentiated hepatocellular carcinomas and modulates sorafenib response in *in vitro* models

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Running title: HOXA13 expression discriminates high-grade HCC

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ABSTRACT

Hepatocellular carcinoma (HCC) represents the fifth and ninth cause of mortality among male and female cancer patients, respectively and typically arises on a background of a cirrhotic liver. HCC develops in a multi-step process, often encompassing chronic liver injury, steatosis and cirrhosis eventually leading to the malignant transformation of hepatocytes. Aberrant expression of the class I homeobox gene family (HOX), a group of genes crucial in embryogenesis, has been reported in a variety of malignancies including solid tumors. Among HOX genes, *HOXA13* is most overexpressed in HCC and is known to be directly regulated by the long non-coding RNA *HOTTIP*. In this study, taking advantage of a tissue microarray containing 305 tissue specimens, we found that HOXA13 protein expression increased monotonically from normal liver to cirrhotic liver to HCC and that HOXA13-positive HCCs were preferentially poorly differentiated and had fewer E-cadherin-positive cells. In two independent cohorts, patients with HOXA13-positive HCC had worse overall survival than those with HOXA13-negative HCC. Using HOXA13 immunohistochemistry and *HOTTIP* RNA *in situ* hybridization on consecutive sections of 16 resected HCCs, we demonstrated that HOXA13 and *HOTTIP* were expressed in the same neoplastic hepatocyte populations. Stable overexpression of HOXA13 in liver cancer cell lines resulted in increased colony formation on soft agar and migration potential as well as reduced sensitivity to sorafenib *in vitro*. Our results provide compelling evidence of a role for HOXA13 in HCC development and highlight for the first time its ability to modulate response to sorafenib.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the fifth and ninth cause of mortality among male and female cancer patients, respectively[1, 2] and typically arises on a background of a cirrhotic liver.[3] HCC develops in a multi-step process, often encompassing chronic liver injury, steatosis and cirrhosis eventually leading to the malignant transformation of hepatocytes.[4] HCC is unique among cancers, in that no standard first-line cytotoxic therapy is routinely used in the clinic [5] and limited therapeutic choices are available.[6, 7] Currently, the multi-tyrosine kinase inhibitor sorafenib is the only option for HCCs in advanced stages. Of note, there are no validated predictive biomarkers for sorafenib response in HCC.[8]

Class I homeobox gene family (HOX) consists of a group of 39 highly conserved transcription factors known for their role as master regulators of embryonic development.[9] The HOX genes are distributed over 4 chromosomal regions each containing between 9 and 11 members that aligned into 13 paralogous groups.[10, 11] During developmental phases, HOX genes regulate the formation of distinct anatomical regions, through the maintenance of spatio-temporal collinearity across the anterior-posterior body axis.[12-14] By contrast, in adult tissues, HOX genes maintain homeostasis by preserving the coordinates established during embryonic growth.[15, 16]

Aberrant expression of the HOX genes has been reported in a variety of malignancies including solid tumors.[17-21] Among HOX genes, *HOXA13* has been reported to be the most deregulated in HCC.[22-25] An earlier study found that knocking down HOXA13 reduces anchorage-dependent growth *in vitro* and cancer cell growth in mice with esophageal squamous cell carcinoma.[26] Moreover, upregulation of HOXA13 has been linked to the activation of the Wnt and TGF-β pathways,[21] suggesting that HOXA13 may play a role in determining the aggressiveness of HCCs. Furthermore, we recently reported that HOXA13 is directly regulated by the long non-coding RNA (lncRNA) HOXA transcript at the distal tip (*HOTTIP*), located in physical contiguity with *HOXA13*, via the interaction with the WDR5/MLL complex.[22] Notably, HOXA13 and *HOTTIP* co-expression was found to be associated with disease progression, metastasis formation and worse clinical outcome,[22] thus suggesting that the molecular axis controlled by HOXA13 and *HOTTIP* plays a pivotal role in HCC progression.[22]

In this study, we sought to determine whether HOXA13 is expressed at the protein level and whether its expression is associated with prognosis. We also evaluated the effect of HOXA13 on sorafenib response *in vitro* using stable liver cancer cell lines with either overexpression or knockdown of HOXA13.

MATERIALS AND METHODS

Tissue microarray data and patient samples

Tissue microarray (TMA) was constructed from formalin-fixed and paraffin-embedded (FFPE) specimens using a custom-built instrument (Beecher Instruments, Silver Spring, MD) as previously described.[27] Specimens were mostly collected from patients with early stage disease who were eligible for and underwent surgical resection without prior treatment. The TMA contained 434 tissue specimens of which 305 were suitable for evaluation, which included 115 HCC, 82 cirrhotic liver and 108 normal liver samples (Supplementary Table S1). Exclusion criteria from the analysis were the absence of tissue punch, poor HOXA13 immunostaining quality or the lack of comprehensive clinical data. Tissue samples were retrieved from the archives of the Institute of Pathology, University Hospital Basel, Switzerland. Histologic grading and classification of HCCs and non-neoplastic liver tissue samples was performed by two experienced pathologists with an expertise in liver pathology (M.S.M. and L.M.T.) according to the World Health Organization (WHO) guidelines[28] and the Edmondson & Steiner grading system.[29] Additionally, samples were classified using the Barcelona Clinic Liver Cancer (BCLC) staging classification.[30] A second cohort of samples comprising whole sections from 43 resected HCC samples from sorafenib-treated patients was obtained from the Unit of Pathology, University of Milan and Humanitas Clinical and Research Center, Milan, Italy (Supplementary Table S2). Histologic grading and classification of this cohort of samples was performed by three experienced pathologists (C.N., M.R. and L.M.T.) as described for the TMA cohort. The study was approved by the institutional review boards of the respective authors' institutions.

Immunohistochemistry

Immunohistochemical (IHC) staining for HOXA13 and E-cadherin was performed on 4 µm sections of FFPE tissue using primary antibodies against HOXA13 (Abcam, Cambridge, UK; clone ab106503, dilution 1:200) and E-cadherin (Cell Marque, Rocklin, CA; clone EP000Y, ready-to-use). Specifically, sections were pre-treated with CC1 (Ventana Medical Systems, Tucson, AZ) and incubated with primary antibodies against HOXA13 and E-cadherin. Staining procedures were performed on a Benchmark immunohistochemistry

staining system (Ventana Medical Systems, Tucson, AZ) using iVIEW-DAB as chromogen as previously described.[31] Positive and negative controls were included in each run. Immunoreactivity was scored by two experienced pathologists (M.S.M. and L.M.T.) For HOXA13, TMA specimens (tissue punches) were analyzed by evaluating the staining intensity (0-3, Figure 1a). For HOXA13 on whole tissue sections using at least two non-consecutive fields, both the staining intensity (0-3) and the percentage of stained cells (i.e. hepatocytes) were evaluated as described by Allred *et al*.[32] and classified as HOXA13-high and HOXA13-low using a semi-quantitative score accounting for both staining intensity and the percentage of stained cells using the following formula:

$$((-x_0*25) + (x_1*20+ x_2*30+ x_3*50)*75))/100$$

where x_i is the percentage of cells with intensity i. Sample with positive and negative scores were classified as HOXA13-high and HOXA13-low, respectively.

Analysis of putative HOXA13 binding sites on *CDH1* promoter

The promoter sequence of the human *CDH1* gene (encoding E-cadherin, Unigene ID: Hs.461086, ID of transcript: NM_004360) was retrieved from the DataBase of Transcriptional Start Site (DBTSS, http://dbtss-old.hgc.jp/hg19_mm9/), encompassing the genomic regions 2000bp upstream to 400bp downstream of the *CDH1* transcriptional start site. The *CDH1* promoter sequence was scanned for the presence of putative binding sites of HOXA13 using the JASPAR algorithm[33] (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and the frequency matrix model (MA0650.1) corresponding to the human HOXA13 (Supplementary Figure S1a). The default relative score cut-off of 0.8 was used. Based on the results from JASPAR, a graphical representation of the distribution of putative binding sites of HOXA13 on the promoter region of *CDH1* gene was obtained using ConSite service (http://consite.genereg.net/).

HOTTIP RNA *in situ* hybridization

Single color RNA *in situ* hybridization (ISH) was performed using the RNAscope® 2.0 HD Red Chromogenic Reagent Kit (Advanced Cell Diagnostics, Newark, CA) per the manufacturer's guidelines. Paired double-Z oligonucleotide probes were designed against Hs-HOTTIP RNA (Catalog number 400133, Advanced Cell Diagnostics, Newark, CA) using custom software as described previously.[34] GenBank accession numbers, number of probe pairs, and probe regions are: NR_037843, 20 pairs, nucleotides 388-1386. 4 µm sections of FFPE tissue were incubated at 60°C for 1 hr prior to use. After de-paraffinization

and dehydration, the tissues were air-dried and treated with peroxidase blocker before boiling at 100-104°C in a pre-treatment solution for 15 min. Protease was then applied at 40°C for 30 min. Target probes were hybridized at 40°C for 2 hrs. All hybridizations were performed in a HybEZ system (Advanced Cell Diagnostics, Newark, CA) at 40°C. Hybridization signals were detected by chromogenic precipitate development and RNA staining signal was identified as red dots. Following the RNAscope assay, samples were counterstained for 2 min with 50% Gill's Hematoxylin diluted in dH_2O. Each sample was quality controlled for RNA integrity with a probe specific to the housekeeping gene cyclophilin B (*PPIB*); only samples with an average of >4 dots per cell were included for analysis. Negative control background staining was evaluated using a probe specific to the bacterial *dapB* gene. To verify that the RNAscope method was performed with technical accuracy, references slides consisting of FFPE HeLa cells were tested with *PPIB* and *dapB* in parallel with tissue samples. Staining intensity was scored on the 4-point scale (i.e. 0-3) by two experienced pathologists (M.S.M and L.M.T.) as for HOXA13 IHC staining intensity.

Cell lines maintenance and generation of stable clones

Liver cancer-derived cell lines SNU-449 and HepG2 were obtained from the Laboratory of Experimental Carcinogenesis (Bethesda, MD), authenticated by short tandem repeat profiling as previously described[35] and tested for mycoplasma infection using a PCR-based test (ATCC). All cell lines were maintained under the condition as recommended by the provider. Total RNA and proteins were extracted from cells at 75% confluence using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacture's guidelines. SNU-449 and HepG2 cells were transfected as described by Wang *et al.*[36] to generate stable HOXA13-expressing cell lines.

Stable HOXA13 knockdown cells were obtained using the TALEN. Specifically, $13x10^4$ cells were plated in 6-well plate and after 24 hr cells were transfected with lipofectamine3000 (Thermo Fisher Scientific, Reinach, CH) according to the manufacturer's procedure with pcDNA3.1+ containing the full-length human cDNA of HOXA13 and empty vector as control.[22] The cells were selected with 50 ug/ml of G418 (Gibco) for 72 hrs. For the knocking down of HOXA13, SNU-449 and HepG2 were simultaneously transfected with TAL2209/2209, a gift from Keith Joung (plasmids # 36672 and #36671, Addgene, Cambridge, MA).[37] 24 hrs after transfection, cells were plated in 96-well plate using a dilution of 1 cell/well.

Relative expression of *HOXA13* by qRT-PCR

cDNA was synthesized from 1 µg of total RNA using Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). *HOXA13* expression was assessed using the TaqMan® Probe-Based Gene Expression Analysis (Applied Biosystems, Foster City, CA; probe Hs00426284_m1). Measurements were normalized using *GAPDH* and *18S* levels as reference. The fold change in gene expression was calculated using the standard ΔΔCt method as previously described.[23, 38] All reverse-transcriptase reactions, including no-template controls, were run on an Applied Biosystem 7900HT thermocycler. All samples were analyzed in triplicate.

Soft-agar colony assay, proliferation assay and *in vitro* sorafenib exposure

Soft agar colony formation assay was performed as previously described.[39] Specifically, 10^3 cells were plated in 60mm dishes in a solution containing Dulbecco's modified Eagle's medium 2X (Thermo Fisher Scientific, Foster City, CA), TPB Buffer (Thermo Fisher Scientific) and 1.25% of Noble Agar (Difco, Franklin Lakes, NJ). Cells were harvested and counted, and a layer of 7ml solution containing Noble Agar was left to polymerize on the bottom of the dishes. Meanwhile cells were diluted in 2ml of the same solution, plated and left growing for 2 weeks in the incubator. Pictures of four different fields were taken using an inverted microscope and cell colonies were counted and measured in size using the ImageJ software (Version 1.49). Colonies were classified into three groups based on thresholds defined using the size distribution (arbitrary units) of the control group as small (first quartile), medium (2^{nd} and 3^{rd} quartiles) and large (4^{th} quartile).

Cell proliferation was assayed using the xCELLigence system (RTCA, ACEA Biosciences, San Diego, CA). Background impedance of the xCELLigence system was measured for 12 sec using 50 µL of room temperature cell culture media in each well of E-plate 16. SNU-449 and HepG2 cells were grown and expanded in tissue culture flasks as previously described.[40] After reaching 75% confluence, the cells were washed with PBS and detached from the flasks using a short treatment with trypsin/EDTA. 10,000 cells were dispensed into each well of an E-plate 16. Growth and proliferation of the cells were monitored every 15 min up to 72 hrs via the incorporated sensor electrode arrays of the xCELLigence system, using the RTCA-integrated software according to the manufacturer's parameters.

In vitro sorafenib exposure experiments were carried out as described by Rothweiler *et al*.[41] Briefly, cells were grown as above and 24 hrs after cell seeding, sorafenib at final concentration of 10 µM or 20 µM or DMSO (0.1% v/v) was added. Cell growth was assayed for up to 72 hours. Data analysis and data normalization to the time before sorafenib/ DMSO addition was carried out using the RTCA Software 1.2.1.1002. Cell index values from 3 replicate experiments were normalized to DMSO, which was set as 100%.

Migration Assay.

Migration assay was performed with CIM plates using the xCELLigence technologies (OLS OMNI Life Science, Basel, Switzerland). Stably transfected cells were detached and counted. $3x10^4$ cells were plated in each well according to manufacturer's instruction and migration was assessed 12, 24 and 36 hrs after seeding.

Statistical analysis

For statistical analysis, the Chi-squared test (χ^2 test) and Fisher's exact test for categorical variables and Mann-Whitney U/multiple t-tests/t-tests for continuous variables were used. Patient survival was assessed using the Kaplan–Meier method and analyzed using the log-rank test. Correlation tests were performed using Spearman correlation. All tests were two-sided and *P*-values ≤0.05 were considered statistically significant. Analyses were performed using GraphPad 6.0 (Prism, La Jolla, CA) and SPSS (version 24, IBM, San Francisco, CA).

RESULTS

HOXA13 protein is increased in poorly differentiated and more aggressive HCCs

In a previous study, our group reported an upregulation of the RNA expression levels of the *HOXA13* gene in the majority of HCCs analyzed.[22] To corroborate and to extend this finding to the protein level, we investigated, by means of IHC on a TMA, HOXA13 levels on a large cohort of HCC (n=115), cirrhotic liver (n=108) and normal liver (n=82) specimens (Supplementary Table S1). Samples were evaluated according to their staining intensity (Figure 1a). Our analysis revealed that HOXA13 was expressed (i.e. intensities 1, 2 or 3) in 10% (8/82) of normal liver, 18% (19/108) of cirrhotic liver and 42% (48/115) of HCC specimens with a monotonic increase from normal liver tissues to cirrhotic tissues to HCCs (*P* <0.001, Chi-squared

test, Figure 1b). Stratification based on the individual HOXA13 IHC intensity scores in HCCs showed that those with high HOXA13 expression (intensity 3) were associated with the highest proportion (29%) of poorly differentiated tumors (*P* <0.001, Chi-squared test, Figure 1c). Altogether, these results highlighted that: i) HOXA13 positivity is more frequent in HCCs compared to normal and cirrhotic livers and ii) high HOXA13 expression is associated with high tumor grade.

Considering our previous finding that *HOXA13* expression was associated with metastasis in HCC,[22] we evaluated the expression of E-cadherin, a master regulation of the metastatic process,[42] in the same set of samples. We observed that HOXA13-positive HCCs had lower percentages of E-cadherin-positive cells compared to HOXA13-negative HCCs, cirrhotic liver and normal liver specimens (mean of positive cells 48% vs 65%, 68% and 69%, respectively, all *P* <0.05, Mann-Whitney U tests, Figure 1d). Stratification of HCCs based on HOXA13 staining intensity scores showed that E-cadherin levels were inversely correlated with HOXA13 expression (all *P* <0.05, Mann-Whitney U tests, Figure 1e).

To determine if there is a causal link between the inverse expression pattern of HOXA13 and E-cadherin, we performed *in silico* and *in vitro* experiments to determine if HOXA13 could directly regulate the expression of E-cadherin (encoded by *CDH1*). Using the JASPAR algorithm[33], we identified several putative HOXA13 binding sites on the *CDH1* promoter sequence, in particular in the region 2000bp to 500bp upstream of the *CDH1* transcriptional start site (Supplementary Figure S1), suggesting that HOXA13 may directly interact with the *CDH1* promoter. Indeed, upon stable overexpression of HOXA13 in the HCC HepG2 cell line, we observed a decrease in E-cadherin and, conversely, an increase in N-cadherin expression (Figure 1f). In line with a previously published report[43], our *in silico* and *in vitro* experiments suggest a possible role for HOXA13 in the regulation of epithelial-to-mesenchymal transition in directly binding to E-cadherin.

Taken together, these results suggest that HOXA13 overexpression is associated with a more undifferentiated and aggressive tumor phenotype in HCC and we provided additional evidences that HOXA13 may regulate E-cadherin.

HOXA13 protein level is associated with HCC patient survival

To explore whether HOXA13 protein levels were associated with the outcome of HCC patients as we previously demonstrated for *HOXA13* mRNA levels,[22] we examined the patient overall survival (OS) and disease-free survival (DFS) for our cohort of TMA HCC samples (n=115, Supplementary Table S1). Patients with HOXA13-positive HCC had shorter OS compared to those with HOXA13-negative disease (median 52 vs 65 months, *P* =0.03, log-rank test, Figure 2a). However, we found no statistical difference in DFS between patients with HOXA13-positive and HOXA13-negative HCC (median 16 vs 20 months, *P* =0.33, log-rank test, Supplementary Figure S2a). Similarly, comparing the OS and DFS stratified according to HOXA13 IHC staining intensities demonstrated that patients with HCCs with the highest IHC intensity (i.e. 3) had the worst OS, but no difference was observed in DFS (*P* =0.04 and *P* =0.24, respectively, log-rank tests, Figure 2b and Supplementary Figure S2b). Focusing on patients with BCLC grade C HCC, since this is the only group eligible for sorafenib treatment, we found that patients with HOXA13-positive HCC had worse OS than those with HOXA13-negative disease (median 20 vs 24 months, *P* =0.05, log-rank test, Figure 2c).

To extend these results to sorafenib-treated patients, we obtained whole tissue sections of an independent cohort composed of late stage sorafenib-treated HCCs (Supplementary Table S2) and classified them as HOXA13-high and –low based on a semi-quantitative score, taking into account the staining intensity and the proportion of positive cells (Methods). In accordance with the results obtained from patients with untreated HCCs, sorafenib-treated patients with HOXA13-positive HCCs showed decreased OS compared to patients with HOXA13-negative disease (median 31 vs 41 months, *P* =0.05, log-rank test, Figure 2d).

These results suggest the HOXA13 protein expression in HCC is associated with OS in patients treated or untreated with sorafenib.

HOXA13 expression overlaps with *HOTTIP* in HCC samples

Based on gene expression array data, we previously showed that *HOXA13* overexpression in HCC is associated with concomitant *HOTTIP* upregulation.[22] To determine whether HOXA13 is upregulated in the same population of cells as *HOTTIP*, we performed HOXA13 IHC and *HOTTIP* RNA *in situ* hybridization (ISH) on consecutive sections of 16 resected HCCs from the sorafenib-treated cohort (Supplementary Table S2). We found that both HOXA13 and *HOTTIP* were heterogeneously expressed in 71% of cases

analyzed. Comparing the expression patterns, we observed that HOXA13 and *HOTTIP* were expressed in virtually the same populations of hepatocytes (Figure 3a) and that the percentages of HOXA13-positive and *HOTTIP*-positive cells demonstrated a significant correlation (r =0.91, P <0.0001, Spearman correlation test, Figure 3b-c).

HOXA13 overexpression enhances cell aggressiveness *in vitro*

In line with the observations that HOXA13 expression is increased in HCCs compared to non-tumoral livers and in poorly differentiated HCCs compared to moderately and well differentiated HCCs, our previous results demonstrated that transient *in vitro* HOXA13 overexpression in liver cancer cells resulted in increased proliferation whereas HOXA13 inhibition strongly reduced cell proliferation.[22] Therefore, we sought to investigate if stable ectopic HOXA13 overexpression would have oncogenic properties in *in vitro* models. We generated liver cancer-derived cell line SNU-449 clones that stably overexpressed HOXA13, as verified by qRT-PCR (Supplementary Figure S3a). To test whether the cells that overexpressed HOXA13 displayed a more aggressive phenotype, we performed a colony formation assay on soft agar and evaluated the number and size of the resulting colonies (Figure 4a). Compared to the untransfected cell lines and the empty vector controls, SNU-449 overexpressing HOXA13 displayed larger colonies (P <0.05, Mann-Whitney U test, Figure 4b) and a higher number of colonies per analyzed field (P <0.05, Mann-Whitney U test, Figure 4c). Additionally, we performed a migration assay on SNU-449 and HepG2 stably overexpressing HOXA13 and observed that forced expression of *HOXA13* resulted in increased cell migration (both P<0.05, Figure 4 d-e) in both cell lines, thus supporting and corroborating our hypothesis that HOXA13 promotes epithelial-to-mesenchymal transition.

Taken together our results suggest that, in addition to increasing cell proliferation, HOXA13 overexpression also enhances cell aggressiveness and migration *in vitro*.

HOXA13 level modulates sorafenib sensitivity *in vitro*

To provide a functional basis for our observation that sorafenib-treated patients with high HOXA13 levels in their HCCs had worse survival, we sought to investigate the impact of HOXA13 modulation in HCC-derived cell lines upon sorafenib exposure *in vitro*. In addition to the liver cancer-derived cell lines (SNU-449 and HepG2) with stable HOXA13 overexpression using pCMV-vector, we also generated two cell lines (SNU-

449 and HepG2) with HOXA13 knockdown using TALEN, as verified by qRT-PCR (Supplementary Figure S3). To define the appropriate dose of sorafenib *in vitro*, we performed a titration experiment using untransfected SNU-449 and HepG2 (Supplementary Figure S4) and observed a significant inhibition of cell viability at 20 μM sorafenib compared to other concentrations (P <0.05, multiple t-test, Supplementary Figure S4). We then compared the proliferation indices of treated and untreated cells with and without HOXA13 overexpression using the xCelligence system up to 72 hours after sorafenib exposure. In accordance with our previous results,[22] in the absence of sorafenib, HOXA13-overexpressing cells displayed increased cell proliferation compared to the vector control (P <0.01, multiple t-test, Figure 5a/c). By contrast, sorafenib treatment significantly reduced cell proliferation in the vector control but had no effect in HOXA13-overexpressing cells (P <0.01 and P >0.05, respectively, multiple t-tests, Figure 5a/c).

Conversely, when we compared the untreated cells with and without HOXA13 knockdown, HOXA13-knockdown cells had lower cell proliferation than cells with TALEN control in both SNU-449 and HepG2 (both P <0.01, multiple t-tests, Figure 5b/d). On the other hand, upon sorafenib exposure, cell proliferation was significantly reduced in HOXA13 knockdown cells compared to the cells with TALEN control in both cell lines (both P <0.01, multiple t-tests, Figure 5b/d). Overall, these results provide evidence that overexpression of HOXA13 modulates sorafenib response in HCC *in vitro* models.

DISCUSSION

In this study, we reported that HOXA13 protein level increased monotonically from normal liver to cirrhotic liver to HCC. We also observed that HOXA13-positive HCCs were preferentially poorly differentiated and had fewer E-cadherin-positive cells. In addition, we demonstrated that HOXA13 positivity is associated with worse overall survival, but not disease-free survival, which we confirmed in a second cohort consisting of sorafenib-treated patients. Furthermore, we showed that HOXA13 and *HOTTIP* were co-expressed in the same population of neoplastic hepatocytes in HCC. Finally, using liver cancer-derived cell lines, we demonstrated that HOXA13 expression enhances cell aggressiveness and modulates sorafenib response *in vitro*.

Based on gene expression data, we previously reported that *HOXA13* is significantly upregulated in HCC.[22, 23] In this work, by analyzing a large cohort of HCC samples (n=115), we extended our results to the protein

level showing consistent HOXA13 overexpression in HCCs. This observation complements our previous results on the mRNA level,[22, 23] is in line with the findings of another group on the protein level[24] and further highlights that HOXA13 is deregulated in a subgroup of poorly differentiated (i.e. high Edmondson grade) HCCs. Additionally, we observed that HOXA13-positive HCCs harbored fewer E-cadherin-positive cells. The loss of E-cadherin has been linked to the formation of metastases through the process of epithelial-to-mesenchymal transition[44] and several groups recently reported that HOXA13 expression promotes cell invasive properties, for instance in glioma by activating the Wnt and TGF-β pathways.[21] Our observation of HOXA13-positive HCCs displaying a more aggressive phenotype is thus in line with our previously described association between high HOXA13 level and metastatic incidence[22] and the association with worse prognosis in terms of OS in these patients. HOXA13 positivity has been associated with unfavorable survival in HCCs[24] as well as in other tumor entities such as esophageal squamous cell carcinoma,[26, 45] gastric cancer[46] and paediatric acute lymphoblastic leukaemia.[18, 47, 48] In contrast to the previous study in which the authors found associations of HOXA13 expression with both OS and DFS,[24] we found that HOXA13 expression was only associated with unfavorable OS, but not DFS, in two independent cohorts, one of which was a cohort of patients with sorafenib-treated, late-stage disease.

The results from our *in vitro* experiments provide functional evidence for the association observed between HOXA13 overexpression and the more aggressive and less differentiated phenotype in HCCs. We observed loss of E-cadherin upon HOXA13 overexpression and identified putative HOXA13 binding sites in the *CDH1* promoter, suggesting that HOXA13 may have a direct role in the regulation of E-cadherin expression. Additionally, we observed increased colony formation and migration potential in cell lines overexpressing HOXA13, demonstrating that HOXA13 confers a more aggressive phenotype. In terms of sensitivity to sorafenib, forced expression of HOXA13 resulted in reduced sensitivity *in vitro* whereas knockdown of HOXA13 had the opposite effect. Given the role of HOXA13 in regulating E-cadherin and the previously published results[49] suggesting that epithelial-to-mesenchymal transition plays a pivotal role in modulating drug resistance, one can speculate that HOXA13 may affect sensitivity to sorafenib through the regulation of the epithelial-to-mesenchymal transition process. Indeed, the reduced sensitivity to sorafenib may be related to the loss of E-cadherin and hence an epithelial phenotype. Conversely, upregulation of HOXA13 may also affect drug response to multiple drugs in multiple cancer types as HOXA13 overexpression has also been reported to result in gemcitabine resistance in pancreatic cancer.[43] These

results provide a functional basis for the suboptimal response of the subset of patients with HOXA13-positive disease and the observed reduced OS in this group of patients.

Several studies previously demonstrated that *HOXA13* and *HOTTIP* expression are highly correlated.[50-53] Specifically, we reported that *HOTTIP* activates HOXA13 through epigenetic mechanisms including DNA methylation and histone modifications[22] and Wang *et al.* described that, in the Cs12 gastric cells, *HOTTIP* knockdown attenuates the recruitment of WDR5 and MLL1 and promotes DNMT3B localization on the HOXA13 promoter.[50] Here by analyzing HOXA13 IHC and *HOTTIP* ISH on consecutive HCC sections, we showed that HOXA13 and *HOTTIP* displayed intra-tumor heterogeneous pattern of expression and their concomitant overexpression was found in the same populations of transformed hepatocytes. To the best of our knowledge, this is the first *in situ* characterization of the pattern of *HOTTIP* expression in human tissues. Given the increased recognition of the role of lncRNAs in cancer,[54] this observation and the technique are of significant interest. We foresee that *in situ* characterization of *HOTTIP* expression will enable the identification of the specific subpopulations of transformed or malignant cells that express *HOTTIP*.

There are several limitations to our study. Our first and larger cohort of resected samples consisted of TMA punches rather than whole sections, and thus the observed expression may not be representative of the individual tissue samples. To account for intra-tumor heterogeneous expression of HOXA13, for the second cohort for which whole sections were available, we used a semi-quantitative score that accounts for both staining intensity and the percentage of cells for each staining intensity. In addition, the number of patients with BCLC stage C disease and late-stage sorafenib-treated patients for survival analysis was small. It should be noted that, despite the small cohorts, we saw a consistent association of HOXA13 overexpression with worse prognosis in both cohorts. It should also be noted that access to such collections of specimens with the associated outcome data is extremely limited. Finally, our results have not provided mechanistic insight into the association of HOXA13, HOTTIP and E-cadherin expression and the effect of HOXA13 expression on sorafenib sensitivity. Although we found that HOTTIP and HOXA13 are expressed in same population of cells, we have not provided direct evidence to support the hypothesis that HOTTIP regulates HOXA13 expression. Due to the cis-regulatory mechanism of HOTTIP, the physical proximity of HOTTIP to the HOX genes (including HOXA13) on the genome is essential for its regulatory

activity. Indeed, it has been demonstrated by,[36] that ectopic HOTTIP expression driven by retroviral insertion sites scattered randomly throughout the genome is not sufficient to induce 5' HOXA genes. Future experiments using genome editing technology to investigate the regulatory role of HOTTIP would be of great interest.

To conclude, using a combination of clinico-pathologic and protein expression data obtained from two independent cohorts of HCC patients, as well as *in vitro* experiments, we provided evidence for a role of the molecular axis comprising HOXA13/*HOTTIP* in HCC development and response to sorafenib.

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AUTHOR CONTRIBUTIONS

L.M.T. conceived and supervised the study. L.Q. and C.Q. conceived and supervised the molecular experiments; M.S.M., C.N., L.T., M.R. and L.M.T performed pathologic assessment; C.Q., M.L. and P.P. performed molecular experiments. T.P. and L.R. collected patient materials and clinic-pathologic data; L.Q., C.Q., C.C., S.P., C.K.Y.N. and L.M.T. interpreted the results and wrote the manuscript. All authors agreed to the final version of the manuscript.

DISCLOSURE/ CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURES LEGENDS

Figure 1: HOXA13 is expressed in a subset of aggressive HCCs and is inversely correlated with E-cadherin expression. (a) Representative micrographs of HOXA13 IHC with different staining intensities. **(b)** Percentages of HOXA13-positive and-negative normal, cirrhotic and tumoral livers. **(c)** HOXA13 intensity in poorly, moderately and well differentiated HCCs. **(d)** E-cadherin expression levels in normal, cirrhotic, HOXA13-negative and HOXA13-positive tumoral livers**. (e)** E-cadherin expression levels in normal, cirrhotic and tumoral livers according to HOXA13 intensities. **(f)** E-cadherin and N-cadherin protein levels upon overexpression of HOXA13 in HepG2 cell lines. *P*-values ≤0.05 were considered statistically significant. *: *P*-values ≤0.05; Error bars, s.d. of the mean.

Figure 2: High HOXA13 expression is associated with poor overall survival in patients with HCC. Overall survival of 115 HCC patients, **(a)** stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3) or **(b)** stratified according to HOXA13 staining intensities**. (c)** Overall survival of 26 BCLC stage C HCC patients stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3). **(d)** Overall survival of 43 HCC sorafenib-treated patients, stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3). Statistical comparisons were performed using log-rank tests. *P*-values ≤0.05 were considered statistically significant.

Figure 3: HOXA13 and *HOTTIP* expression patterns in HCC. (a) Representative micrographs of HOXA13 IHC and *HOTTIP* ISH stained HCCs. **(b)** Heat map displaying the percentage of HOXA13-positive and *HOTTIP*-positive cells. **(c)** Spearman correlation analysis performed for the percentages of *HOTTIP*-positive and HOXA13-positive cells.

Figure 4: Effect of HOXA13 overexpression on cell transformation in *in vitro* models. (a) Representative micrographs of colonies, **(b)** distribution of sizes of the colonies and **(c)** number of colonies per field in control SNU-449 cells and SNU-449 cells stably overexpressing HOXA13. (**d-e**) Migration potential in SNU-449 and HepG2 cell lines at 12,24 and 36hrs in stable HOXA13 overexpressing cells. All experiments were performed in triplicates. Error bars, s.d. of the mean. Statistical comparisons were

performed using unpaired t-tests with Welch correction. $P \leq 0.05$ were considered statistically significant. *: $P \leq 0.05$; ns: not significant.

Figure 5: HOXA13 modulates sorafenib response in HCC cells. Effect of sorafenib treatment in cells with **(a-c)** stable overexpression of HOXA13 (SNU-449 and HepG2) or **(b-c)** knockdown of HOXA13 (SNU-449 and HepG2) compared to the untransfected controls. Quantification was performed using a spectrophotometer. All experiments were performed in triplicates. Error bars, s.d. of the mean. Statistical comparisons were performed using Holm-Šídák-corrected multiple *t* tests an. $P \leq 0.05$ were considered statistically significant. **: $P \leq 0.01$.

High expression of HOXA13 correlates with poorly differentiated hepatocellular carcinomas and modulates sorafenib response in *in vitro* models

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Running title: HOXA13 expression discriminates high-grade HCC

Abbreviation: Hepatocellular carcinoma (HCC), hepatitis B virus (HBV), hepatitis C virus (HCV), long non-coding RNA (lncRNA), HOTTIP (HOXA transcript at the distal tip).

Keywords: Hepatocellular carcinoma, HOXA13, HOTTIP, sorafenib, tissue microarray.

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ABSTRACT

Hepatocellular carcinoma (HCC) represents the fifth and ninth cause of mortality among male and female cancer patients, respectively and typically arises on a background of a cirrhotic liver. HCC develops in a multi-step process, often encompassing chronic liver injury, steatosis and cirrhosis eventually leading to the malignant transformation of hepatocytes. Aberrant expression of the class I homeobox gene family (HOX), a group of genes crucial in embryogenesis, has been reported in a variety of malignancies including solid tumors. Among HOX genes, *HOXA13* is most overexpressed in HCC and is known to be directly regulated by the long non-coding RNA *HOTTIP*. In this study, taking advantage of a tissue microarray containing 305 tissue specimens, we found that HOXA13 protein expression increased monotonically from normal liver to cirrhotic liver to HCC and that HOXA13-positive HCCs were preferentially poorly differentiated and had fewer E-cadherin-positive cells. In two independent cohorts, patients with HOXA13-positive HCC had worse overall survival than those with HOXA13-negative HCC. Using HOXA13 immunohistochemistry and *HOTTIP* RNA *in situ* hybridization on consecutive sections of 16 resected HCCs, we demonstrated that HOXA13 and *HOTTIP* were expressed in the same neoplastic hepatocyte populations. Stable overexpression of HOXA13 in liver cancer cell lines resulted in increased colony formation on soft agar and migration potential as well as reduced sensitivity to sorafenib *in vitro*. Our results provide compelling evidence of a role for HOXA13 in HCC development and highlight for the first time its ability to modulate response to sorafenib.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the fifth and ninth cause of mortality among male and female cancer patients, respectively[1, 2] and typically arises on a background of a cirrhotic liver.[3] HCC develops in a multi-step process, often encompassing chronic liver injury, steatosis and cirrhosis eventually leading to the malignant transformation of hepatocytes.[4] HCC is unique among cancers, in that no standard first-line cytotoxic therapy is routinely used in the clinic [5] and limited therapeutic choices are available.[6, 7] Currently, the multi-tyrosine kinase inhibitor sorafenib is the only option for HCCs in advanced stages. Of note, there are no validated predictive biomarkers for sorafenib response in HCC.[8]

Class I homeobox gene family (HOX) consists of a group of 39 highly conserved transcription factors known for their role as master regulators of embryonic development.[9] The HOX genes are distributed over 4 chromosomal regions each containing between 9 and 11 members that aligned into 13 paralogous groups.[10, 11] During developmental phases, HOX genes regulate the formation of distinct anatomical regions, through the maintenance of spatio-temporal collinearity across the anterior-posterior body axis.[12-14] By contrast, in adult tissues, HOX genes maintain homeostasis by preserving the coordinates established during embryonic growth.[15, 16]

Aberrant expression of the HOX genes has been reported in a variety of malignancies including solid tumors.[17-21] Among HOX genes, *HOXA13* has been reported to be the most deregulated in HCC.[22-25] An earlier study found that knocking down HOXA13 reduces anchorage-dependent growth *in vitro* and cancer cell growth in mice with esophageal squamous cell carcinoma.[26] Moreover, upregulation of HOXA13 has been linked to the activation of the Wnt and TGF-β pathways,[21] suggesting that HOXA13 may play a role in determining the aggressiveness of HCCs. Furthermore, we recently reported that HOXA13 is directly regulated by the long non-coding RNA (lncRNA) HOXA transcript at the distal tip (*HOTTIP*), located in physical contiguity with *HOXA13*, via the interaction with the WDR5/MLL complex.[22] Notably, HOXA13 and *HOTTIP* co-expression was found to be associated with disease progression, metastasis formation and worse clinical outcome,[22] thus suggesting that the molecular axis controlled by HOXA13 and *HOTTIP* plays a pivotal role in HCC progression.[22]

In this study, we sought to determine whether HOXA13 is expressed at the protein level and whether its expression is associated with prognosis. We also evaluated the effect of HOXA13 on sorafenib response *in vitro* using stable liver cancer cell lines with either overexpression or knockdown of HOXA13.

MATERIALS AND METHODS

Tissue microarray data and patient samples

Tissue microarray (TMA) was constructed from formalin-fixed and paraffin-embedded (FFPE) specimens using a custom-built instrument (Beecher Instruments, Silver Spring, MD) as previously described.[27] Specimens were mostly collected from patients with early stage disease who were eligible for and underwent surgical resection without prior treatment. The TMA contained 434 tissue specimens of which 305 were suitable for evaluation, which included 115 HCC, 82 cirrhotic liver and 108 normal liver samples (Supplementary Table S1). Exclusion criteria from the analysis were the absence of tissue punch, poor HOXA13 immunostaining quality or the lack of comprehensive clinical data. Tissue samples were retrieved from the archives of the Institute of Pathology, University Hospital Basel, Switzerland. Histologic grading and classification of HCCs and non-neoplastic liver tissue samples was performed by two experienced pathologists with an expertise in liver pathology (M.S.M. and L.M.T.) according to the World Health Organization (WHO) guidelines[28] and the Edmondson & Steiner grading system.[29] Additionally, samples were classified using the Barcelona Clinic Liver Cancer (BCLC) staging classification.[30] A second cohort of samples comprising whole sections from 43 resected HCC samples from sorafenib-treated patients was obtained from the Unit of Pathology, University of Milan and Humanitas Clinical and Research Center, Milan, Italy (Supplementary Table S2). Histologic grading and classification of this cohort of samples was performed by three experienced pathologists (C.N., M.R. and L.M.T.) as described for the TMA cohort. The study was approved by the institutional review boards of the respective authors' institutions.

Immunohistochemistry

Immunohistochemical (IHC) staining for HOXA13 and E-cadherin was performed on 4 µm sections of FFPE tissue using primary antibodies against HOXA13 (Abcam, Cambridge, UK; clone ab106503, dilution 1:200) and E-cadherin (Cell Marque, Rocklin, CA; clone EP000Y, ready-to-use). Specifically, sections were pre-treated with CC1 (Ventana Medical Systems, Tucson, AZ) and incubated with primary antibodies against HOXA13 and E-cadherin. Staining procedures were performed on a Benchmark immunohistochemistry

staining system (Ventana Medical Systems, Tucson, AZ) using iVIEW-DAB as chromogen as previously described.[31] Positive and negative controls were included in each run. Immunoreactivity was scored by two experienced pathologists (M.S.M. and L.M.T.) For HOXA13, TMA specimens (tissue punches) were analyzed by evaluating the staining intensity (0-3, Figure 1a). For HOXA13 on whole tissue sections using at least two non-consecutive fields, both the staining intensity (0-3) and the percentage of stained cells (i.e. hepatocytes) were evaluated as described by Allred *et al*.[32] and classified as HOXA13-high and HOXA13-low using a semi-quantitative score accounting for both staining intensity and the percentage of stained cells using the following formula:

$$((-x_0*25) + (x_1*20+ x_2*30+ x_3*50)*75))/100$$

where x_i is the percentage of cells with intensity i. Sample with positive and negative scores were classified as HOXA13-high and HOXA13-low, respectively.

Analysis of putative HOXA13 binding sites on *CDH1* promoter

The promoter sequence of the human *CDH1* gene (encoding E-cadherin, Unigene ID: Hs.461086, ID of transcript: NM_004360) was retrieved from the DataBase of Transcriptional Start Site (DBTSS, http://dbtss-old.hgc.jp/hg19_mm9/), encompassing the genomic regions 2000bp upstream to 400bp downstream of the *CDH1* transcriptional start site. The *CDH1* promoter sequence was scanned for the presence of putative binding sites of HOXA13 using the JASPAR algorithm[33] (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and the frequency matrix model (MA0650.1) corresponding to the human HOXA13 (Supplementary Figure S1a). The default relative score cut-off of 0.8 was used. Based on the results from JASPAR, a graphical representation of the distribution of putative binding sites of HOXA13 on the promoter region of *CDH1* gene was obtained using ConSite service (http://consite.genereg.net/).

HOTTIP RNA *in situ* hybridization

Single color RNA *in situ* hybridization (ISH) was performed using the RNAscope® 2.0 HD Red Chromogenic Reagent Kit (Advanced Cell Diagnostics, Newark, CA) per the manufacturer's guidelines. Paired double-Z oligonucleotide probes were designed against Hs-HOTTIP RNA (Catalog number 400133, Advanced Cell Diagnostics, Newark, CA) using custom software as described previously.[34] GenBank accession numbers, number of probe pairs, and probe regions are: NR_037843, 20 pairs, nucleotides 388-1386. 4 μm sections of FFPE tissue were incubated at 60°C for 1 hr prior to use. After de-paraffinization

and dehydration, the tissues were air-dried and treated with peroxidase blocker before boiling at 100-104°C in a pre-treatment solution for 15 min. Protease was then applied at 40°C for 30 min. Target probes were hybridized at 40°C for 2 hrs. All hybridizations were performed in a HybEZ system (Advanced Cell Diagnostics, Newark, CA) at 40°C. Hybridization signals were detected by chromogenic precipitate development and RNA staining signal was identified as red dots. Following the RNAscope assay, samples were counterstained for 2 min with 50% Gill's Hematoxylin diluted in dH_2O. Each sample was quality controlled for RNA integrity with a probe specific to the housekeeping gene cyclophilin B (*PPIB*); only samples with an average of >4 dots per cell were included for analysis. Negative control background staining was evaluated using a probe specific to the bacterial *dapB* gene. To verify that the RNAscope method was performed with technical accuracy, references slides consisting of FFPE HeLa cells were tested with *PPIB* and *dapB* in parallel with tissue samples. Staining intensity was scored on the 4-point scale (i.e. 0-3) by two experienced pathologists (M.S.M and L.M.T.) as for HOXA13 IHC staining intensity.

Cell lines maintenance and generation of stable clones

Liver cancer-derived cell lines SNU-449 and HepG2 were obtained from the Laboratory of Experimental Carcinogenesis (Bethesda, MD), authenticated by short tandem repeat profiling as previously described[35] and tested for mycoplasma infection using a PCR-based test (ATCC). All cell lines were maintained under the condition as recommended by the provider. Total RNA and proteins were extracted from cells at 75% confluence using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacture's guidelines. SNU-449 and HepG2 cells were transfected as described by Wang *et al.*[36] to generate stable HOXA13-expressing cell lines.

Stable HOXA13 knockdown cells were obtained using the TALEN. Specifically, $13x10^4$ cells were plated in 6-well plate and after 24 hr cells were transfected with lipofectamine3000 (Thermo Fisher Scientific, Reinach, CH) according to the manufacturer's procedure with pcDNA3.1+ containing the full-length human cDNA of HOXA13 and empty vector as control.[22] The cells were selected with 50 ug/ml of G418 (Gibco) for 72 hrs. For the knocking down of HOXA13, SNU-449 and HepG2 were simultaneously transfected with TAL2209/2209, a gift from Keith Joung (plasmids # 36672 and #36671, Addgene, Cambridge, MA).[37] 24 hrs after transfection, cells were plated in 96-well plate using a dilution of 1 cell/well.

Relative expression of *HOXA13* by qRT-PCR

cDNA was synthesized from 1 µg of total RNA using Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). *HOXA13* expression was assessed using the TaqMan® Probe-Based Gene Expression Analysis (Applied Biosystems, Foster City, CA; probe Hs00426284_m1). Measurements were normalized using *GAPDH* and *18S* levels as reference. The fold change in gene expression was calculated using the standard ΔΔCt method as previously described.[23, 38] All reverse-transcriptase reactions, including no-template controls, were run on an Applied Biosystem 7900HT thermocycler. All samples were analyzed in triplicate.

Soft-agar colony assay, proliferation assay and *in vitro* sorafenib exposure

Soft agar colony formation assay was performed as previously described.[39] Specifically, 10^3 cells were plated in 60mm dishes in a solution containing Dulbecco's modified Eagle's medium 2X (Thermo Fisher Scientific, Foster City, CA), TPB Buffer (Thermo Fisher Scientific) and 1.25% of Noble Agar (Difco, Franklin Lakes, NJ). Cells were harvested and counted, and a layer of 7ml solution containing Noble Agar was left to polymerize on the bottom of the dishes. Meanwhile cells were diluted in 2ml of the same solution, plated and left growing for 2 weeks in the incubator. Pictures of four different fields were taken using an inverted microscope and cell colonies were counted and measured in size using the ImageJ software (Version 1.49). Colonies were classified into three groups based on thresholds defined using the size distribution (arbitrary units) of the control group as small (first quartile), medium (2^{nd} and 3^{rd} quartiles) and large (4^{th} quartile).

Cell proliferation was assayed using the xCELLigence system (RTCA, ACEA Biosciences, San Diego, CA). Background impedance of the xCELLigence system was measured for 12 sec using 50 µL of room temperature cell culture media in each well of E-plate 16. SNU-449 and HepG2 cells were grown and expanded in tissue culture flasks as previously described.[40] After reaching 75% confluence, the cells were washed with PBS and detached from the flasks using a short treatment with trypsin/EDTA. 10,000 cells were dispensed into each well of an E-plate 16. Growth and proliferation of the cells were monitored every 15 min up to 72 hrs via the incorporated sensor electrode arrays of the xCELLigence system, using the RTCA-integrated software according to the manufacturer's parameters.

In vitro sorafenib exposure experiments were carried out as described by Rothweiler *et al*.[41] Briefly, cells were grown as above and 24 hrs after cell seeding, sorafenib at final concentration of 10 µM or 20 µM or DMSO (0.1% v/v) was added. Cell growth was assayed for up to 72 hours. Data analysis and data normalization to the time before sorafenib/ DMSO addition was carried out using the RTCA Software 1.2.1.1002. Cell index values from 3 replicate experiments were normalized to DMSO, which was set as 100%.

Migration Assay.

Migration assay was performed with CIM plates using the xCELLigence technologies (OLS OMNI Life Science, Basel, Switzerland). Stably transfected cells were detached and counted. $3x10^4$ cells were plated in each well according to manufacturer's instruction and migration was assessed 12, 24 and 36 hrs after seeding.

Statistical analysis

For statistical analysis, the Chi-squared test (χ^2 test) and Fisher's exact test for categorical variables and Mann-Whitney U/multiple t-tests/t-tests for continuous variables were used. Patient survival was assessed using the Kaplan–Meier method and analyzed using the log-rank test. Correlation tests were performed using Spearman correlation. All tests were two-sided and *P*-values ≤0.05 were considered statistically significant. Analyses were performed using GraphPad 6.0 (Prism, La Jolla, CA) and SPSS (version 24, IBM, San Francisco, CA).

RESULTS

HOXA13 protein is increased in poorly differentiated and more aggressive HCCs

In a previous study, our group reported an upregulation of the RNA expression levels of the *HOXA13* gene in the majority of HCCs analyzed.[22] To corroborate and to extend this finding to the protein level, we investigated, by means of IHC on a TMA, HOXA13 levels on a large cohort of HCC (n=115), cirrhotic liver (n=108) and normal liver (n=82) specimens (Supplementary Table S1). Samples were evaluated according to their staining intensity (Figure 1a). Our analysis revealed that HOXA13 was expressed (i.e. intensities 1, 2 or 3) in 10% (8/82) of normal liver, 18% (19/108) of cirrhotic liver and 42% (48/115) of HCC specimens with a monotonic increase from normal liver tissues to cirrhotic tissues to HCCs (*P* <0.001, Chi-squared

test, Figure 1b). Stratification based on the individual HOXA13 IHC intensity scores in HCCs showed that those with high HOXA13 expression (intensity 3) were associated with the highest proportion (29%) of poorly differentiated tumors (P <0.001, Chi-squared test, Figure 1c). Altogether, these results highlighted that: i) HOXA13 positivity is more frequent in HCCs compared to normal and cirrhotic livers and ii) high HOXA13 expression is associated with high tumor grade.

Considering our previous finding that *HOXA13* expression was associated with metastasis in HCC,[22] we evaluated the expression of E-cadherin, a master regulation of the metastatic process,[42] in the same set of samples. We observed that HOXA13-positive HCCs had lower percentages of E-cadherin-positive cells compared to HOXA13-negative HCCs, cirrhotic liver and normal liver specimens (mean of positive cells 48% vs 65%, 68% and 69%, respectively, all P <0.05, Mann-Whitney U tests, Figure 1d). Stratification of HCCs based on HOXA13 staining intensity scores showed that E-cadherin levels were inversely correlated with HOXA13 expression (all P <0.05, Mann-Whitney U tests, Figure 1e).

To determine if there is a causal link between the inverse expression pattern of HOXA13 and E-cadherin, we performed *in silico* and *in vitro* experiments to determine if HOXA13 could directly regulate the expression of E-cadherin (encoded by *CDH1*). Using the JASPAR algorithm[33], we identified several putative HOXA13 binding sites on the *CDH1* promoter sequence, in particular in the region 2000bp to 500bp upstream of the *CDH1* transcriptional start site (Supplementary Figure S1), suggesting that HOXA13 may directly interact with the *CDH1* promoter. Indeed, upon stable overexpression of HOXA13 in the HCC HepG2 cell line, we observed a decrease in E-cadherin and, conversely, an increase in N-cadherin expression (Figure 1f). In line with a previously published report[43], our *in silico* and *in vitro* experiments suggest a possible role for HOXA13 in the regulation of epithelial-to-mesenchymal transition in directly binding to E-cadherin.

Taken together, these results suggest that HOXA13 overexpression is associated with a more undifferentiated and aggressive tumor phenotype in HCC and we provided additional evidences that HOXA13 may regulate E-cadherin.

HOXA13 protein level is associated with HCC patient survival

To explore whether HOXA13 protein levels were associated with the outcome of HCC patients as we previously demonstrated for *HOXA13* mRNA levels,[22] we examined the patient overall survival (OS) and disease-free survival (DFS) for our cohort of TMA HCC samples (n=115, Supplementary Table S1). Patients with HOXA13-positive HCC had shorter OS compared to those with HOXA13-negative disease (median 52 vs 65 months, *P* =0.03, log-rank test, Figure 2a). However, we found no statistical difference in DFS between patients with HOXA13-positive and HOXA13-negative HCC (median 16 vs 20 months, *P* =0.33, log-rank test, Supplementary Figure S2a). Similarly, comparing the OS and DFS stratified according to HOXA13 IHC staining intensities demonstrated that patients with HCCs with the highest IHC intensity (i.e. 3) had the worst OS, but no difference was observed in DFS (*P* =0.04 and *P* =0.24, respectively, log-rank tests, Figure 2b and Supplementary Figure S2b). Focusing on patients with BCLC grade C HCC, since this is the only group eligible for sorafenib treatment, we found that patients with HOXA13-positive HCC had worse OS than those with HOXA13-negative disease (median 20 vs 24 months, *P* =0.05, log-rank test, Figure 2c).

To extend these results to sorafenib-treated patients, we obtained whole tissue sections of an independent cohort composed of late stage sorafenib-treated HCCs (Supplementary Table S2) and classified them as HOXA13-high and –low based on a semi-quantitative score, taking into account the staining intensity and the proportion of positive cells (Methods). In accordance with the results obtained from patients with untreated HCCs, sorafenib-treated patients with HOXA13-positive HCCs showed decreased OS compared to patients with HOXA13-negative disease (median 31 vs 41 months, *P* =0.05, log-rank test, Figure 2d).

These results suggest the HOXA13 protein expression in HCC is associated with OS in patients treated or untreated with sorafenib.

HOXA13 expression overlaps with *HOTTIP* in HCC samples

Based on gene expression array data, we previously showed that *HOXA13* overexpression in HCC is associated with concomitant *HOTTIP* upregulation.[22] To determine whether HOXA13 is upregulated in the same population of cells as *HOTTIP*, we performed HOXA13 IHC and *HOTTIP* RNA *in situ* hybridization (ISH) on consecutive sections of 16 resected HCCs from the sorafenib-treated cohort (Supplementary Table S2). We found that both HOXA13 and *HOTTIP* were heterogeneously expressed in 71% of cases

analyzed. Comparing the expression patterns, we observed that HOXA13 and *HOTTIP* were expressed in virtually the same populations of hepatocytes (Figure 3a) and that the percentages of HOXA13-positive and *HOTTIP*-positive cells demonstrated a significant correlation (r =0.91, P <0.0001, Spearman correlation test, Figure 3b-c).

HOXA13 overexpression enhances cell aggressiveness *in vitro*

In line with the observations that HOXA13 expression is increased in HCCs compared to non-tumoral livers and in poorly differentiated HCCs compared to moderately and well differentiated HCCs, our previous results demonstrated that transient *in vitro* HOXA13 overexpression in liver cancer cells resulted in increased proliferation whereas HOXA13 inhibition strongly reduced cell proliferation.[22] Therefore, we sought to investigate if stable ectopic HOXA13 overexpression would have oncogenic properties in *in vitro* models. We generated liver cancer-derived cell line SNU-449 clones that stably overexpressed HOXA13, as verified by qRT-PCR (Supplementary Figure S3a). To test whether the cells that overexpressed HOXA13 displayed a more aggressive phenotype, we performed a colony formation assay on soft agar and evaluated the number and size of the resulting colonies (Figure 4a). Compared to the untransfected cell lines and the empty vector controls, SNU-449 overexpressing HOXA13 displayed larger colonies (P <0.05, Mann-Whitney U test, Figure 4b) and a higher number of colonies per analyzed field (P <0.05, Mann-Whitney U test, Figure 4c). Additionally, we performed a migration assay on SNU-449 and HepG2 stably overexpressing HOXA13 and observed that forced expression of *HOXA13* resulted in increased cell migration (both P<0.05, Figure 4 d-e) in both cell lines, thus supporting and corroborating our hypothesis that HOXA13 promotes epithelial-to-mesenchymal transition.

Taken together our results suggest that, in addition to increasing cell proliferation, HOXA13 overexpression also enhances cell aggressiveness and migration *in vitro*.

HOXA13 level modulates sorafenib sensitivity *in vitro*

To provide a functional basis for our observation that sorafenib-treated patients with high HOXA13 levels in their HCCs had worse survival, we sought to investigate the impact of HOXA13 modulation in HCC-derived cell lines upon sorafenib exposure *in vitro*. In addition to the liver cancer-derived cell lines (SNU-449 and HepG2) with stable HOXA13 overexpression using pCMV-vector, we also generated two cell lines (SNU-

449 and HepG2) with HOXA13 knockdown using TALEN, as verified by qRT-PCR (Supplementary Figure S3). To define the appropriate dose of sorafenib *in vitro*, we performed a titration experiment using untransfected SNU-449 and HepG2 (Supplementary Figure S4) and observed a significant inhibition of cell viability at 20 μM sorafenib compared to other concentrations (P <0.05, multiple t-test, Supplementary Figure S4). We then compared the proliferation indices of treated and untreated cells with and without HOXA13 overexpression using the xCelligence system up to 72 hours after sorafenib exposure. In accordance with our previous results,[22] in the absence of sorafenib, HOXA13-overexpressing cells displayed increased cell proliferation compared to the vector control (P <0.01, multiple t-test, Figure 5a/c). By contrast, sorafenib treatment significantly reduced cell proliferation in the vector control but had no effect in HOXA13-overexpressing cells (P <0.01 and P >0.05, respectively, multiple t-tests, Figure 5a/c).

Conversely, when we compared the untreated cells with and without HOXA13 knockdown, HOXA13-knockdown cells had lower cell proliferation than cells with TALEN control in both SNU-449 and HepG2 (both P <0.01, multiple t-tests, Figure 5b/d). On the other hand, upon sorafenib exposure, cell proliferation was significantly reduced in HOXA13 knockdown cells compared to the cells with TALEN control in both cell lines (both P <0.01, multiple t-tests, Figure 5b/d). Overall, these results provide evidence that overexpression of HOXA13 modulates sorafenib response in HCC *in vitro* models.

DISCUSSION

In this study, we reported that HOXA13 protein level increased monotonically from normal liver to cirrhotic liver to HCC. We also observed that HOXA13-positive HCCs were preferentially poorly differentiated and had fewer E-cadherin-positive cells. In addition, we demonstrated that HOXA13 positivity is associated with worse overall survival, but not disease-free survival, which we confirmed in a second cohort consisting of sorafenib-treated patients. Furthermore, we showed that HOXA13 and *HOTTIP* were co-expressed in the same population of neoplastic hepatocytes in HCC. Finally, using liver cancer-derived cell lines, we demonstrated that HOXA13 expression enhances cell aggressiveness and modulates sorafenib response *in vitro*.

Based on gene expression data, we previously reported that *HOXA13* is significantly upregulated in HCC.[22, 23] In this work, by analyzing a large cohort of HCC samples (n=115), we extended our results to the protein

level showing consistent HOXA13 overexpression in HCCs. This observation complements our previous results on the mRNA level,[22, 23] is in line with the findings of another group on the protein level[24] and further highlights that HOXA13 is deregulated in a subgroup of poorly differentiated (i.e. high Edmondson grade) HCCs. Additionally, we observed that HOXA13-positive HCCs harbored fewer E-cadherin-positive cells. The loss of E-cadherin has been linked to the formation of metastases through the process of epithelial-to-mesenchymal transition[44] and several groups recently reported that HOXA13 expression promotes cell invasive properties, for instance in glioma by activating the Wnt and TGF-β pathways.[21] Our observation of HOXA13-positive HCCs displaying a more aggressive phenotype is thus in line with our previously described association between high HOXA13 level and metastatic incidence[22] and the association with worse prognosis in terms of OS in these patients. HOXA13 positivity has been associated with unfavorable survival in HCCs[24] as well as in other tumor entities such as esophageal squamous cell carcinoma,[26, 45] gastric cancer[46] and paediatric acute lymphoblastic leukaemia.[18, 47, 48] In contrast to the previous study in which the authors found associations of HOXA13 expression with both OS and DFS,[24] we found that HOXA13 expression was only associated with unfavorable OS, but not DFS, in two independent cohorts, one of which was a cohort of patients with sorafenib-treated, late-stage disease.

The results from our *in vitro* experiments provide functional evidence for the association observed between HOXA13 overexpression and the more aggressive and less differentiated phenotype in HCCs. We observed loss of E-cadherin upon HOXA13 overexpression and identified putative HOXA13 binding sites in the *CDH1* promoter, suggesting that HOXA13 may have a direct role in the regulation of E-cadherin expression. Additionally, we observed increased colony formation and migration potential in cell lines overexpressing HOXA13, demonstrating that HOXA13 confers a more aggressive phenotype. In terms of sensitivity to sorafenib, forced expression of HOXA13 resulted in reduced sensitivity *in vitro* whereas knockdown of HOXA13 had the opposite effect. Given the role of HOXA13 in regulating E-cadherin and the previously published results[49] suggesting that epithelial-to-mesenchymal transition plays a pivotal role in modulating drug resistance, one can speculate that HOXA13 may affect sensitivity to sorafenib through the regulation of the epithelial-to-mesenchymal transition process. Indeed, the reduced sensitivity to sorafenib may be related to the loss of E-cadherin and hence an epithelial phenotype. Conversely, upregulation of HOXA13 may also affect drug response to multiple drugs in multiple cancer types as HOXA13 overexpression has also been reported to result in gemcitabine resistance in pancreatic cancer.[43] These

results provide a functional basis for the suboptimal response of the subset of patients with HOXA13-positive disease and the observed reduced OS in this group of patients.

Several studies previously demonstrated that *HOXA13* and *HOTTIP* expression are highly correlated.[50-53] Specifically, we reported that *HOTTIP* activates HOXA13 through epigenetic mechanisms including DNA methylation and histone modifications[22] and Wang *et al.* described that, in the Cs12 gastric cells, *HOTTIP* knockdown attenuates the recruitment of WDR5 and MLL1 and promotes DNMT3B localization on the HOXA13 promoter.[50] Here by analyzing HOXA13 IHC and *HOTTIP* ISH on consecutive HCC sections, we showed that HOXA13 and *HOTTIP* displayed intra-tumor heterogeneous pattern of expression and their concomitant overexpression was found in the same populations of transformed hepatocytes. To the best of our knowledge, this is the first *in situ* characterization of the pattern of *HOTTIP* expression in human tissues. Given the increased recognition of the role of lncRNAs in cancer,[54] this observation and the technique are of significant interest. We foresee that *in situ* characterization of *HOTTIP* expression will enable the identification of the specific subpopulations of transformed or malignant cells that express *HOTTIP*.

There are several limitations to our study. Our first and larger cohort of resected samples consisted of TMA punches rather than whole sections, and thus the observed expression may not be representative of the individual tissue samples. To account for intra-tumor heterogeneous expression of HOXA13, for the second cohort for which whole sections were available, we used a semi-quantitative score that accounts for both staining intensity and the percentage of cells for each staining intensity. In addition, the number of patients with BCLC stage C disease and late-stage sorafenib-treated patients for survival analysis was small. It should be noted that, despite the small cohorts, we saw a consistent association of HOXA13 overexpression with worse prognosis in both cohorts. It should also be noted that access to such collections of specimens with the associated outcome data is extremely limited. Finally, our results have not provided mechanistic insight into the association of HOXA13, HOTTIP and E-cadherin expression and the effect of HOXA13 expression on sorafenib sensitivity. Although we found that HOTTIP and HOXA13 are expressed in same population of cells, we have not provided direct evidence to support the hypothesis that HOTTIP regulates HOXA13 expression. Due to the cis-regulatory mechanism of HOTTIP, the physical proximity of HOTTIP to the HOX genes (including HOXA13) on the genome is essential for its regulatory

activity. Indeed, it has been demonstrated by,[36] that ectopic HOTTIP expression driven by retroviral insertion sites scattered randomly throughout the genome is not sufficient to induce 5' HOXA genes. Future experiments using genome editing technology to investigate the regulatory role of HOTTIP would be of great interest.

To conclude, using a combination of clinico-pathologic and protein expression data obtained from two independent cohorts of HCC patients, as well as *in vitro* experiments, we provided evidence for a role of the molecular axis comprising HOXA13/*HOTTIP* in HCC development and response to sorafenib.

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AUTHOR CONTRIBUTIONS

L.M.T. conceived and supervised the study. L.Q. and C.Q. conceived and supervised the molecular experiments; M.S.M., C.N., L.T., M.R. and L.M.T performed pathologic assessment; C.Q., M.L. and P.P. performed molecular experiments. T.P. and L.R. collected patient materials and clinic-pathologic data; L.Q., C.Q., C.C., S.P., C.K.Y.N. and L.M.T. interpreted the results and wrote the manuscript. All authors agreed to the final version of the manuscript.

DISCLOSURE/ CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURES LEGENDS

Figure 1: HOXA13 is expressed in a subset of aggressive HCCs and is inversely correlated with E-cadherin expression. (a) Representative micrographs of HOXA13 IHC with different staining intensities. **(b)** Percentages of HOXA13-positive and-negative normal, cirrhotic and tumoral livers. **(c)** HOXA13 intensity in poorly, moderately and well differentiated HCCs. **(d)** E-cadherin expression levels in normal, cirrhotic, HOXA13-negative and HOXA13-positive tumoral livers**. (e)** E-cadherin expression levels in normal, cirrhotic and tumoral livers according to HOXA13 intensities. **(f)** E-cadherin and N-cadherin protein levels upon overexpression of HOXA13 in HepG2 cell lines. *P*-values ≤0.05 were considered statistically significant. *: *P*-values ≤0.05; Error bars, s.d. of the mean.

Figure 2: High HOXA13 expression is associated with poor overall survival in patients with HCC. Overall survival of 115 HCC patients, **(a)** stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3) or **(b)** stratified according to HOXA13 staining intensities**. (c)** Overall survival of 26 BCLC stage C HCC patients stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3). **(d)** Overall survival of 43 HCC sorafenib-treated patients, stratified into HOXA13-negative (staining intensity 0) and HOXA13-positive (staining intensity 1/2/3). Statistical comparisons were performed using log-rank tests. *P*-values ≤0.05 were considered statistically significant.

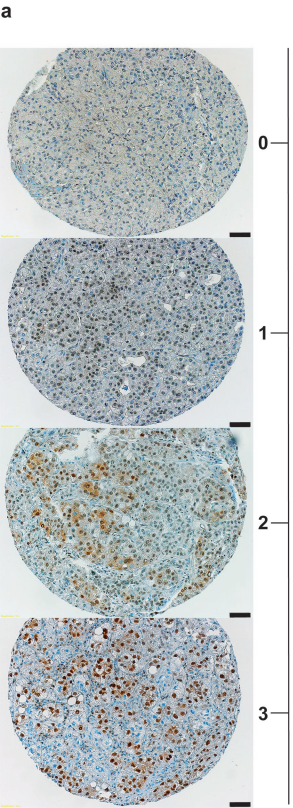
Figure 3: HOXA13 and *HOTTIP* expression patterns in HCC. (a) Representative micrographs of HOXA13 IHC and *HOTTIP* ISH stained HCCs. **(b)** Heat map displaying the percentage of HOXA13-positive and *HOTTIP*-positive cells. **(c)** Spearman correlation analysis performed for the percentages of *HOTTIP*-positive and HOXA13-positive cells.

Figure 4: Effect of HOXA13 overexpression on cell transformation in *in vitro* models. (a) Representative micrographs of colonies, **(b)** distribution of sizes of the colonies and **(c)** number of colonies per field in control SNU-449 cells and SNU-449 cells stably overexpressing HOXA13. (**d-e**) Migration potential in SNU-449 and HepG2 cell lines at 12,24 and 36hrs in stable HOXA13 overexpressing cells. All experiments were performed in triplicates. Error bars, s.d. of the mean. Statistical comparisons were

performed using unpaired t-tests with Welch correction. $P \leq 0.05$ were considered statistically significant. *: $P \leq 0.05$; ns: not significant.

Figure 5: HOXA13 modulates sorafenib response in HCC cells. Effect of sorafenib treatment in cells with **(a-c)** stable overexpression of HOXA13 (SNU-449 and HepG2) or **(b-c)** knockdown of HOXA13 (SNU-449 and HepG2) compared to the untransfected controls. Quantification was performed using a spectrophotometer. All experiments were performed in triplicates. Error bars, s.d. of the mean. Statistical comparisons were performed using Holm-Šídák-corrected multiple *t* tests an. $P \leq 0.05$ were considered statistically significant. **: $P \leq 0.01$.

Figure 1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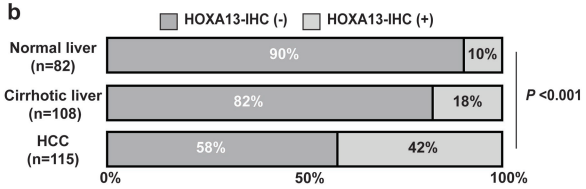
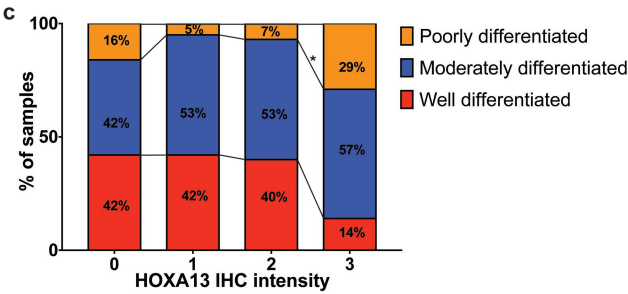
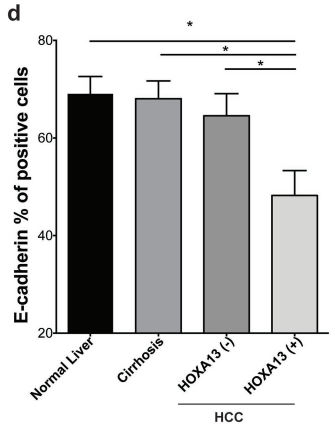
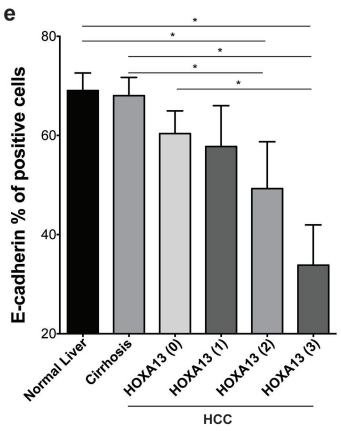

b
HOXA13-IHC (-)
HOXA13-IHC (+)
Normal liver (n=82)
90%
10%
Cirrhotic liver (n=108)
82%
18%
HCC (n=115)
58%
42%
P <0.001
0%
50%
100%


c
Poorly differentiated
Moderately differentiated
Well differentiated
% of samples
100
50
0
16%
42%
42%
5%
53%
42%
7%
53%
40%
29%
57%
14%
*
0
1
2
3
HOXA13 IHC intensity


d
E-cadherin % of positive cells
80
60
40
20
*
*
*
Normal Liver
Cirrhosis
HOXA13 (-)
HOXA13 (+)
HCC


e
E-cadherin % of positive cells
80
60
40
20
*
*
*
*
*
Normal Liver
Cirrhosis
HOXA13 (0)
HOXA13 (1)
HOXA13 (2)
HOXA13 (3)
HCC

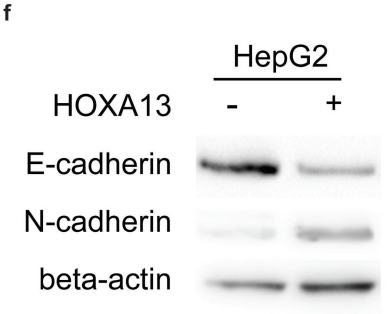

f
HepG2
HOXA13
-
+
E-cadherin
N-cadherin
beta-actin

Figure 2

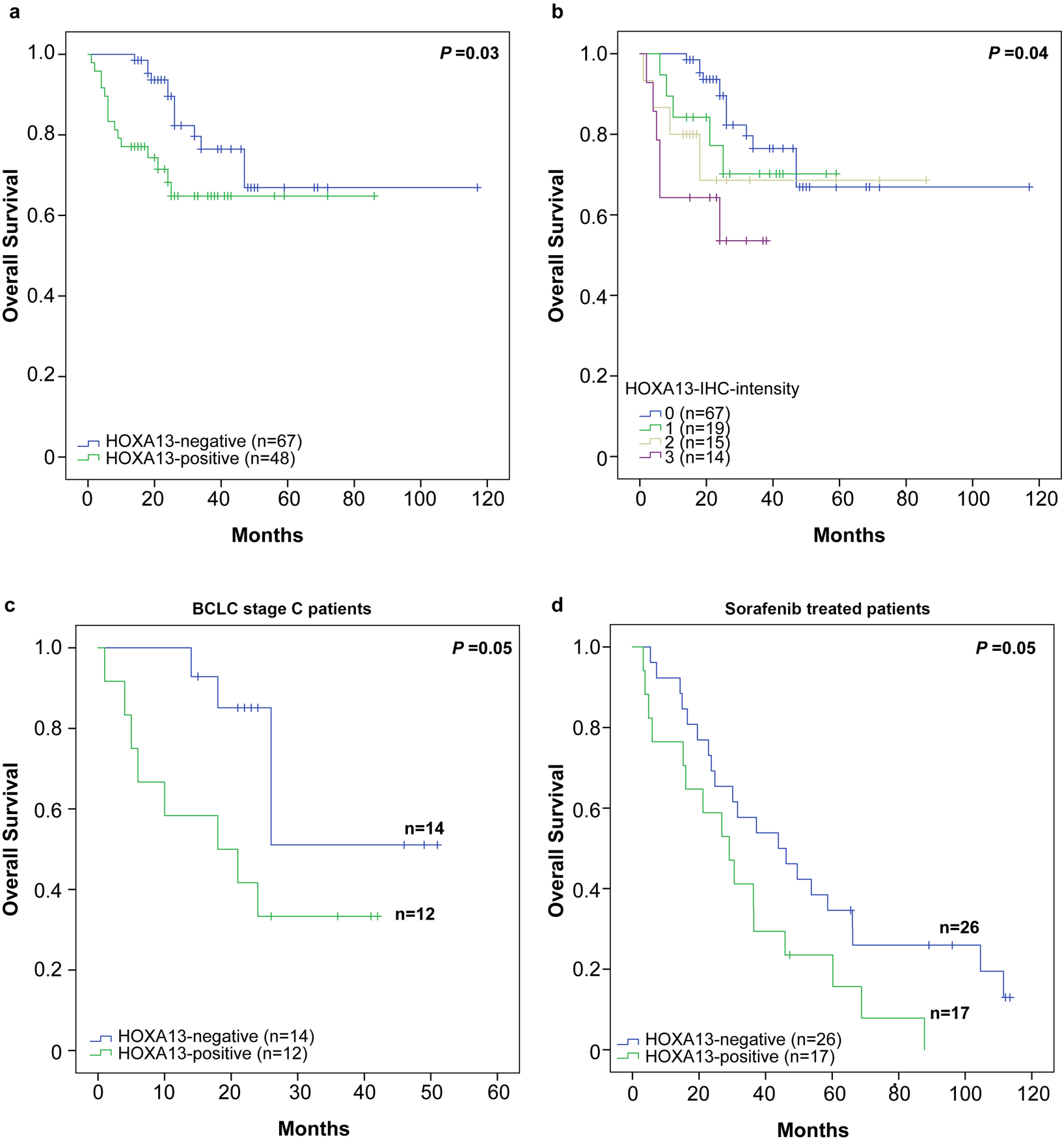

a
P =0.03
Overall Survival
1.0
0.8
0.6
0.4
0.2
0
HOXA13-negative (n=67)
HOXA13-positive (n=48)
0
20
40
60
80
100
120
Months
b
P =0.04
Overall Survival
HOXA13-IHC-intensity
0 (n=67)
1 (n=19)
2 (n=15)
3 (n=14)
Months
c
BCLC stage C patients
P =0.05
Overall Survival
n=14
n=12
HOXA13-negative (n=14)
HOXA13-positive (n=12)
0
10
20
30
40
50
60
Months
d
Sorafenib treated patients
P =0.05
Overall Survival
n=26
n=17
HOXA13-negative (n=26)
HOXA13-positive (n=17)
Months

Figure 3

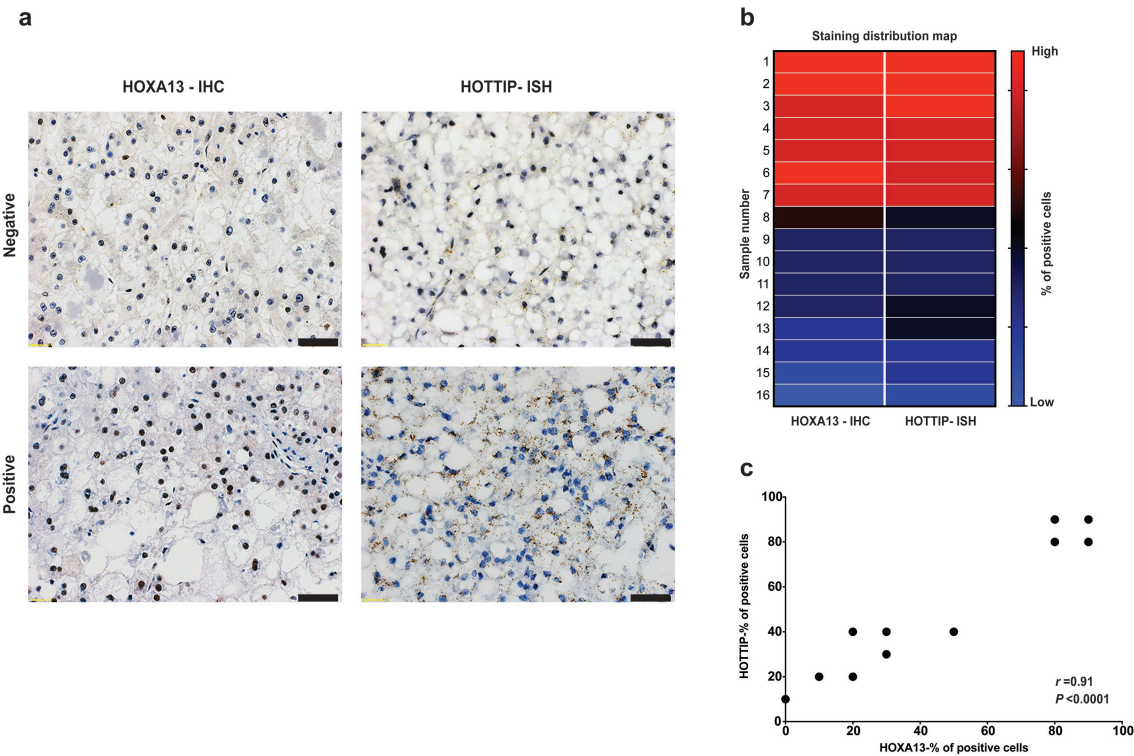

a
HOXA13 - IHC
HOTTIP- ISH
Negative
Positive
b
Staining distribution map
Sample number
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
High
Low
% of positive cells
HOXA13 - IHC
HOTTIP- ISH
c
HOTTIP-% of positive cells
HOXA13-% of positive cells
0
20
40
60
80
100
r =0.91
P <0.0001

Figure 4

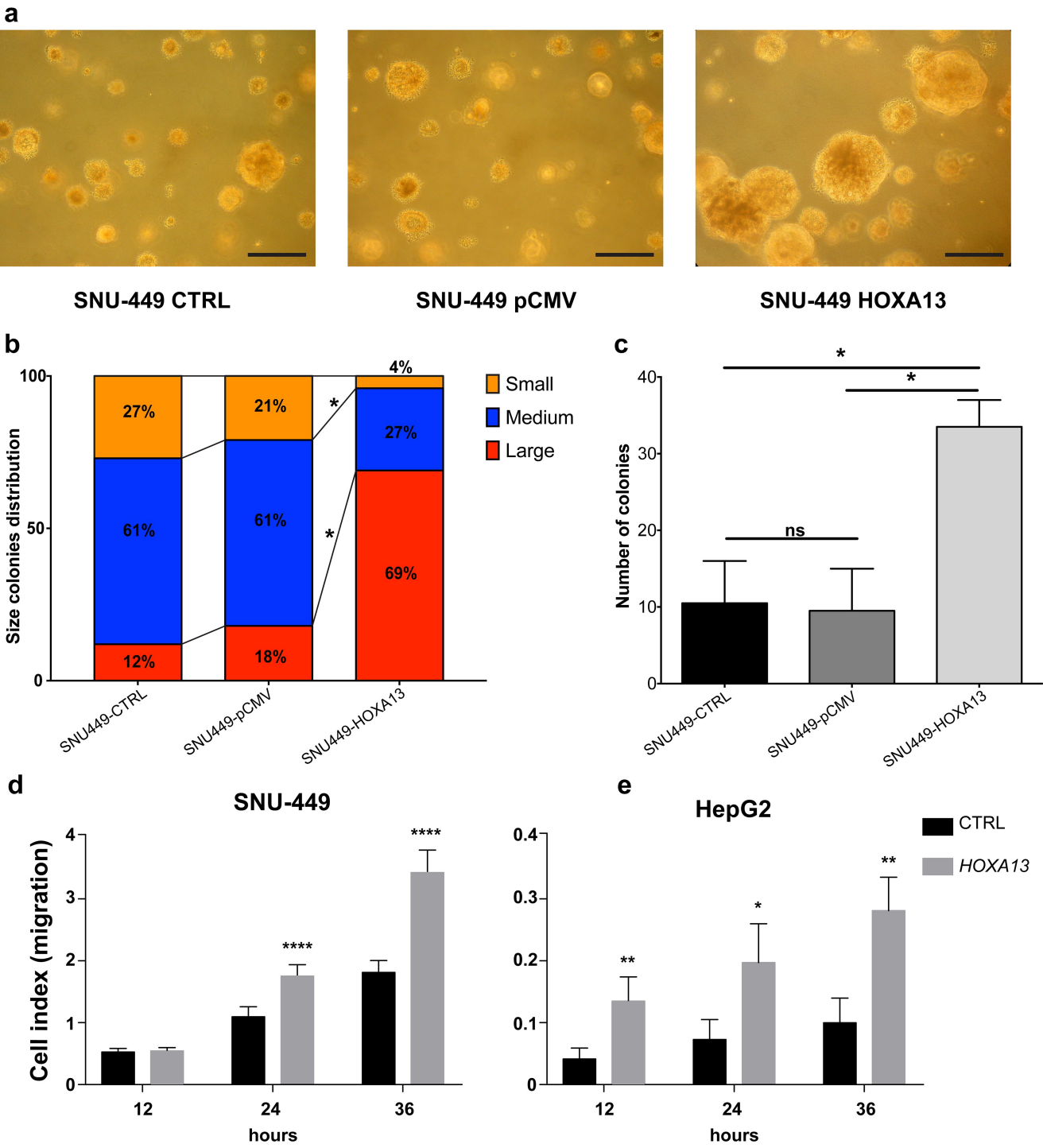
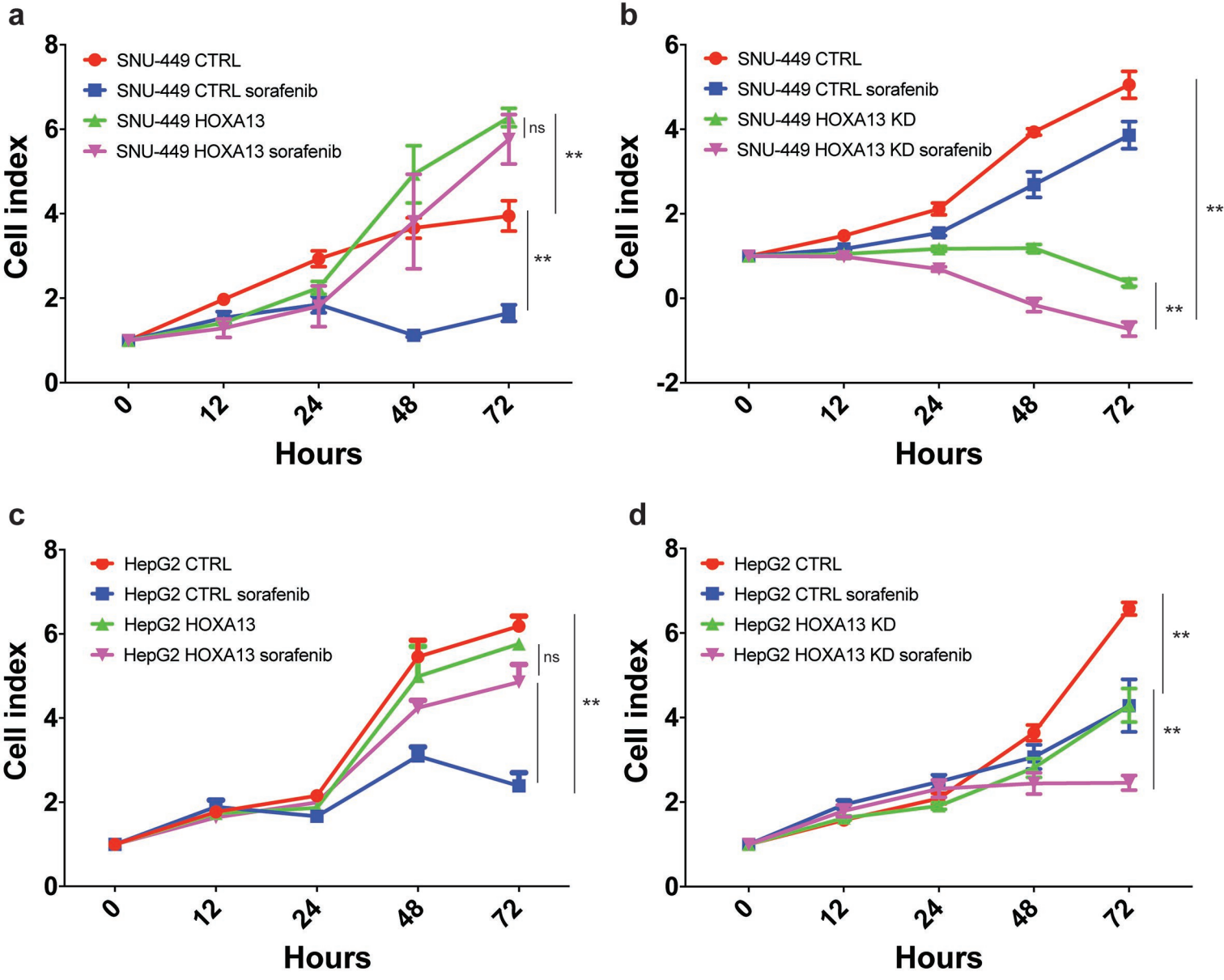

a
SNU-449 CTRL
SNU-449 pCMV
SNU-449 HOXA13
b
Size colonies distribution
100
50
0
27%
61%
12%
21%
61%
18%
4%
27%
69%
*
*
Small
Medium
Large
SNU449-CTRL
SNU449-pCMV
SNU449-HOXA13
c
Number of colonies
40
30
20
10
0
*
*
ns
SNU449-CTRL
SNU449-pCMV
SNU449-HOXA13
d
SNU-449
Cell index (migration)
4
3
2
1
0
****
****
12
24
36
hours
e
HepG2
CTRL
HOXA13
0.4
0.3
0.2
0.1
0
**
*
**
12
24
36
hours

Figure 5


a
SNU-449 CTRL
SNU-449 CTRL sorafenib
SNU-449 HOXA13
SNU-449 HOXA13 sorafenib
Cell index
Hours
ns
**
**
b
SNU-449 CTRL
SNU-449 CTRL sorafenib
SNU-449 HOXA13 KD
SNU-449 HOXA13 KD sorafenib
Cell index
Hours
**
**
c
HepG2 CTRL
HepG2 CTRL sorafenib
HepG2 HOXA13
HepG2 HOXA13 sorafenib
Cell index
Hours
ns
**
d
HepG2 CTRL
HepG2 CTRL sorafenib
HepG2 HOXA13 KD
HepG2 HOXA13 KD sorafenib
Cell index
Hours
**
**