Supplemental information

Label-retaining liver cancer cells are relatively resistant to sorafenib

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Abbreviations:

ABC	ATP binding cassette
ACAN	Aggrecan
ACTC1	Cardiac muscle alpha actin 1
Akt	V-akt murine thymoma viral oncogene homolog
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
BAD	BCL2-associated agonist of cell death
BCL2L1	BCL2-like 1
B-RAF	V-raf murine sarcoma viral oncogene homolog b1
BTRC	Beta transducin repeat containing
CASP	Caspase
CCND2	Cyclin D2
Cdc42	Cell-division-cycle-42
c-Kit	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
C-Raf	V-raf murine leukemia viral oncogene homolog
CSC	Cancer stem cells
CTNNA1	Catenin-A1
Ct	Cross threshold
Cy5	Cyanine-5
dUTP	Deoxyuridinetriphosphate
EIF2S1	Eukaryotic translation initiation factor 2S1
ERK	Extracellular-signal-regulated kinases

FGF3	Fibroblast growth factor 3
FLT	Fms-related tyrosine kinase
FOXA2	Forkhead box A2
GJB1	Gap junction protein B1
HCC	Hepatocellular carcinoma
IEF	Isoelectric focusing
IPA	Ingenuity pathway analysis
ISL1	ISL LIM homeobox 1
LRC	Label retaining cells
LRCC	Label retaining cancer cells
MEK	Mitogen-activated protein kinase kinase
mTOR	Mammalian target of rapamycin
MYST2	MYST histone acetyltransferase 2
Notch2	Notch homolog 2
NUMB	Numb homolog
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinases
Pygo1	Pygopus homolog 1
qRT-PCR	Real-time quantitative reverse-transcription polymerase-chain-reaction
Raf	V-raf murine leukemia viral oncogene homolog
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1
Ras	Rat sarcoma
RET	Ret proto-oncogene
RTK	Receptor Tyrosine Kinsae
RB1	Retinoblastoma 1
SCL	Basic-helix-loop-helix transcription factor SCL
SD	Standard Deviation
SEM	Standard Error of Mean
SHC1	Src homology 2 domain containing transforming protein 1
SHARP	Sorafenib HCC assessment randomized protocol
SOX1	Sex-determining-region-Y-box-1
STG	Sorafenib target genes
STP	Sorafenib target proteins
TIC	Tumor initiating cells
TKI	Tyrosine kinase inhibitor
TUBB3	Beta tubulin 3
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VIM	Vimentin
WNT	Wingless type MMTV integration site family

Supplemental Table 1. All the HCC cell lines were composed of LRCC and non-LRCC

Cell line	LRCC	Non-LRCC
PLC/PRF/5	1.3% - 1.7%	98.3% - 98.7%
HuH-7	4.1% - 5.1%	94.9% - 95.9%
SK-Hep-1	0.9%	99.1%

Supplemental Table 2. There were no inherent differences between LRCC and non-LRCC in terms of susceptibility to apoptosis or toxicity in the absence of sorafenib treatment. The numbers represent fold changes in viability, apoptosis and toxicity of LRCC vs. non-LRCC.

		Fold change (Mean)	Fold Change (SEM)	P value (t test, n=3)
Viability	PLC/PRF/5	1.342065	0.972946	0.53029
	HuH-7	-1.11181	1.382265	0.708622
	SK-Hep-1	-1.27741	1.075053	0.795428
	All	1.25312	1.062673	0.479547
Apoptosis	PLC/PRF/5	1.102616	0.883373	0.886871
	HuH-7	1.956538	0.890297	0.101728
	SK-Hep-1	-2.13869	0.732624	0.286647
	All	-1.33563	0.91919	0.747327
Toxicity	PLC/PRF/5	1.293842	0.939976	0.59048
	HuH-7	-1.03935	1.599585	0.682412
	SK-Hep-1	-1.13505	1.264102	0.198056
	All	-1.14565	0.822784	0.492999

Supplementary Table 3. MEK-ERK-AKT relative protein levels in LRCC and non-LRCC without treatment with sorafenib [p indicated mono-phosphorylated; pp indicated double-phosphorylated]

Protein	PLC/PRF/5 (RLU)			HUH-7 (Peak %)		
	LRCC	Non-LRCC	P value (t test)	LRCC	Non-LRCC	P value (t test)
MEK1 (5.57)	7.0 ± 0.3	10.4 ± 0.7	0.011	2.3 ± 0.2	4.6 ± 0.1	0.00043
MEK1 (5.65)	5.6 ± 0.3	4.9 ± 0.5	0.31	5.5 ± 0.5	5.2 ± 0.2	0.57
MEK1 (5.70)	16.5 ± 0.5	22.0 ± 1.4	0.021	7.5 ± 0.5	10.8 ± 0.2	0.0030
MEK2 (5.60)	8.9 ± 1.2	9.8 ± 1.3	0.65	3.3 ± 0.4	6.6 ± 0.6	0.014
MEK2 (5.83)	47.0 ± 1.4	55.7 ± 3.2	0.067	38.9 ± 1.5	48.4 ± 3.1	0.72
MEK2 (6.0)	9.7 ± 0.8	10.0 ± 1.1	0.85	7.5 ± 0.3	6.0 ± 0.53	0.088
MEK1/2pS218-222 (5.70)	2.1 ± 0.6	2.5 ± 0.4	0.60	2.9 ± 0.3	3.4 ± 0.4	0.36
MEK2pS218-222 (5.83)	2.0 ± 0.3	4.4 ± 0.7	0.035	1.2 ± 0.2	2.1 ± 0.2	0.043
MEK1pS218-222 (5.84)	1.3 ± 0.4	2.6 ± 0.5	0.12	0.4 ± 0.05	0.6 ± 0.06	0.043
MEK1/2pS218-222 (5.91)	3.7 ± 0.6	3.9 ± 0.7	0.85	5.0 ± 0.8	6.6 ± 0.5	0.17
MEK1pS218-222 (6.13)	2.8 ± 0.5	4.6 ± 0.8	0.11	1.3 ± 0.3	1.9 ± 0.2	0.16
MEK2pT292 (5.57)	10.1 ± 0.7	6.4 ± 0.9	0.037	3.8 ± 0.67	5.4 ± 0.5	0.13
MEK2pT292 (5.65)	6.0 ± 0.3	8.0 ± 1.0	0.13	3.5 ± 0.4	4.7 ± 0.17	0.057
MEK2pT292 (5.70)	6.5 ± 0.7	5.0 ± 0.7	0.21	6.2 ± 1.0	5.0 ± 0.5	0.34
MEK2pT292 (5.84)	10.1 ± 0.6	12.6 ± 1.7	0.24	9.1 ± 0.8	14.4 ± 1.2	0.024
MEK1pT386 (5.57)	17.0 ± 2.8	11.1 ± 2.6	0.20	9.5 ± 0.7	7.8 ± 0.8	0.20
MEK1pT386 (5.65)	11.1 ± 0.3	13.6 ± 1.2	0.11	3.6 ± 0.4	6.6 ± 0.2	0.0015
MEK1pT386 (5.70)	27.5 ± 1.0	28.8 ± 3.0	0.70	18.1 ± 1.9	17.7 ± 2.7	0.90
MEK1pT386 (5.84)	68.0 ± 2.4	89.9 ± 6.2	0.030	28.9 ± 2.1	46.5 ± 3.7	0.015
ppERK1	239.7 ± 22.4	5226.9 ± 873.5	0.0046	368.6 ± 18.6	1229.1 ± 178.0	0.0086
pERK1	5108.6 ± 1004.1	2023.1 ± 345.7	0.043	26.4 ± 13.5	5.0 ± 4.7	0.40
ppERK2	2478.38 ± 651.2	11672.8 ± 2512.5	0.024	2353.2 ± 359.3	1599.3 ± 383.1	0.31
pERK2	234.29 ± 63.5	3459.7 ± 801.1	0.016	0.05 ± 0.01	16.6 ± 2.7	0.0037
AKT1 (5.26)	9.1 ± 8.6	117.0 ± 33.8	0.037	0.2 ± 0.2	6.5 ± 0.6	0.00064
AKT1 (5.32)	1096.4 ± 33.8	1138.0 ± 54.2	0.90	2.7 ± 0.1	29.0 ± 1.7	0.0001
AKT1 (5.43)	1098.8 ± 55.9	1534.7 ± 113.8	0.034	37.1 ± 1.5	30.2 ± 1.0	0.018
AKT1 (5.52)	1707.6 ± 45.5	2415.4 ± 124.4	0.0078	8.5 ± 0.5	24.7 ± 1.3	0.00033
AKT1 (5.59)	779.6 ± 52.1	974.8 ± 107.6	0.23	7.7 ± 0.4	13.8 ± 0.7	0.0016
AKT2 (5.68)	189.6 ± 26.8	275.3 ± 51.0	0.24	7.7 ± 0.5	7.7 ± 0.7	0.95
AKT2 (5.84)	423.8 ± 105.8	734.2 ± 174.2	0.22	12.2 ± 1.4	13.7 ± 1.7	0.54

Supplementary Table 4. Gene expression affected by sorafenib in different HCC cell lines. Numbers in bold show statistically significant changes. P value was calculated by two-tailed t test.

Gene group	Comparison	Gene	PLC/PRF/5		HuH-7		SK-Hep-1	
			Fold	p (n=3)	Fold	p (n=3)	Fold	p (n=3)
Sorafenib target genes (STG)	LRCC vs. non-LRCC treated with sorafenib	**BCL2L1**	-1.3	6.6e-5	**2.0e6**	**3.5e-4**	**2.0**	**0.0001**
		FLT4	**2.4**	**0.013**	8.3	0.055	**4.3**	**4e-5**
		VIM	1.1	0.090	-1.2	0.88	**-3.8e6**	**1e-5**
	LRCC treated with vs. without sorafenib	**ALDH1A1**	**4.1e6**	**2e-6**	3.1e4	0.16	1.3	0.037
		EIF2S1	-1.1	0.38	**-14.9**	**7.4e-4**	**-7.8e4**	**0.0031**
Wnt pathway genes	LRCC vs. non-LRCC treated with sorafenib	**CCND2**	2.1	0.36	14.4	0.053	**3.5**	**0.0073**
		WNT9A	1.8	0.014	2.3	0.52	**7.5**	**0.0048**
	LRCC treated with vs. without sorafenib	**PGYO1**	1.6	0.40	**15.7**	**0.0019**	-1.0	0.75
		WNT16	-5.0	0.074	**-30.1**	**0.0063**	-1.1	0.84
Stem cell genes	LRCC vs. non-LRCC treated with sorafenib	**FGF3**	**-69.5**	**0.0069**	-2.6	0.23	-1.2	0.38
		GJB1	**-2.9**	**3.0e-4**	**-57.0**	**6.2e-4**	1.0	0.49
		ISL1	**-114.3**	**0.0056**	**-859.6**	**9.9e-5**	**-2.2**	**0.018**
		MYST2	-1.5	0.0052	**-2.2e5**	**4.3e-5**	**-3.6**	**0.0047**
		NUMB	1.6	0.0091	**-1.9e5**	**1e-5**	**-3.0**	**0.011**
	LRCC treated with vs. without sorafenib	**ACTC1**	**-208.4**	**1.0e-4**	-4.9	0.41	**-30.7**	**5e-5**
		ACAN	-3.2	0.089	**-91.7**	**0.0075**	**-416.5**	**0.017**
		BTRC	-1.8	0.0016	**-74.9**	**2.0e-4**	**-3.5**	**0.011**
		CDC42	**-2.8**	**3.4e-5**	**-18.9**	**1.8e-4**	-1.5	0.041
		CTNNA1	**-2.2**	**4.4e-4**	**-2.7e4**	**2.9e-5**	-2.4	0.0014
		FGF3	**-263.2**	**0.041**	-6.4	0.19	**-12.8**	**1.0e-5**
		FOXA2	**-4.1**	**1.8e-4**	-1.8e5	1.1e-4	-2.2	0.095
		GJB1	**-4.6**	**2.9e-5**	**-69.8**	**8.6e-5**	-2.3e3	0.17
		NOTCH2	-2.7	0.16	**-1.5e5**	**3.3e-5**	**-7.5**	**0.018**
		RB1	**-2.8**	**1.3e-4**	**-30.2**	**3.0e-4**	**-4.1**	**0.0012**
		SOX1	**-484.6**	**0.0010**	-1.8	0.079	-1.9e3	3.0e-5
		TUBB3	-1.8	9.3e-4	**-8.1**	**0.0035**	**-3.0**	**1.3e-4**

Supplemental Note

RTK: Receptor Tyrosine Kinsae

Ras: Rat sarcoma, V-Ha-ras Harvey rat sarcoma viral oncogene homolog, small GTPases.

Raf: V-raf murine leukemia viral oncogene homolog, serine/threonine-specific protein kinases.

MEK: Mitogen-Activated Protein Kinase Kinase, threonine and tyrosine kinases.

ERK: Extracellular-signal-regulated kinases, Mitogen-activated protein kinase

PI3K: Phosphatidylinositol 3-kinases.

AKT: Protein Kinase B (PKB), RAC-alpha serine/threonine-protein kinases.

mTOR: Mammalian Target of Rapamycin, a 289-kDa serine/threonine protein kinase.

SHC1: (Src homology 2 domain containing) transforming protein 1. It's an adapter protein in signal transduction pathways, linking activated receptor tyrosine kinases to the Ras pathway by recruitment of the GRB2/SOS complex.

SCL: The basic helix-loop-helix (bHLH) transcription factor SCL (also known as TAL1).

FLT4 encodes for VEGF-C, involved in lymphangiogenesis, and interact with SHC1 and SCL to suppress apoptosis and induce cell proliferation via ERK. (1)

Supplemental Materials and Methods:

MEK, ERK and AKT protein kinase analysis

Antibodies used for immunoprobing: Anti-phospho-Erk (1:50, Cell Signaling, Danvers, MA, http://www.cellsignal.com), anti-Erk1/2 (1:300, Millipore, Billerica, MA, http://www.millipore.com), anti-MEK1 (1:200, Millipore), anti-MEK2 (1:100, Epitomics, Burlingame, CA, http://www.epitomics.com), anti-AKTpan (1:50, Cell Signaling), anti-MEKpS218/222 (1:50, Epitomics), anti-MEKpT292 (1:50, Millipore), anti-MEKpT386 (1:50, Novus, Littleton, CO, http://www.novusbio.com), anti-PKC alpha (1:50, Santa-Cruz, Santa Cruz, CA, http://www.scbt.com), anti-PKC delta (1:100, Santa-Cruz), anti-HSP70 (as an internal control, 1:300, Novus) and anti-Alas1 (as an internal control, 1:50, Abcam, Cambridge, MA, http://www.abcam.com). Digital images were analyzed and quantified with Compass software (ProteinSimple).

Gene expression: Real-time qRT-PCR

Live LRCC and non-LRCC cells were isolated and RNA was extracted according to manufacturer's protocol (QIAGEN, Valencia, CA, http://www.qiagen.com). Real-time qRT-PCR for customized SuperArrays were done in triplicates following the manufacturer's protocol (SABiosciences, Frederick, MD, http://www.sabiosciences.com).

For pre-amplification of cDNA target templates, we used the Nano PreAmp PCR kit as per manufacturer's protocol: 95ºC for 10 minutes, 12 cycles of 95ºC/15 seconds and 60ºC/2 minutes. After PCR, tubes were put on ice. 2μl of the side reaction reducer (SR1) was then added to each pre-amplified reaction, incubated at 37ºC for 15 minutes, and followed by heat inactivation at 95ºC for 5 minutes. RNase-DNase free water was then added to each of nano PreAMP PCR reaction to adjust for volume.

Real-time qPCR was done using the SABioscience RT^2 master mix in a 384 wells plate for both customized sorafenib target genes SuperArray, or Human Wnt pathway and stem cells genes SuperArrays using ABI 7900 HT system (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com) following the supplier's protocol.

Gene expression: Data analysis

Ct values were analyzed using the SABioscience software (SABiosciences, Valencia, CA, http://www.sabiosciences.com). More than 2 fold-regulations were considered above the technical error threshold.

Gene expression: Pathway analysis

Analyses were done using Ingenuity Pathway Analysis software (IPA 9.0, Ingenuity Systems, Inc., Redwood City, CA, http://www.ingenuity.com).

Supplemental Reference

1. Martin R, Lahlil R, Damert A, Miquerol L, Nagy A, Keller G, Hoang T. SCL interacts with VEGF to suppress apoptosis at the onset of hematopoiesis. Development 2004;131:693-702.