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Overexpression of Mortalin in hepatocellular carcinoma and its relationship with angiogenesis and epithelial to mesenchymal transition

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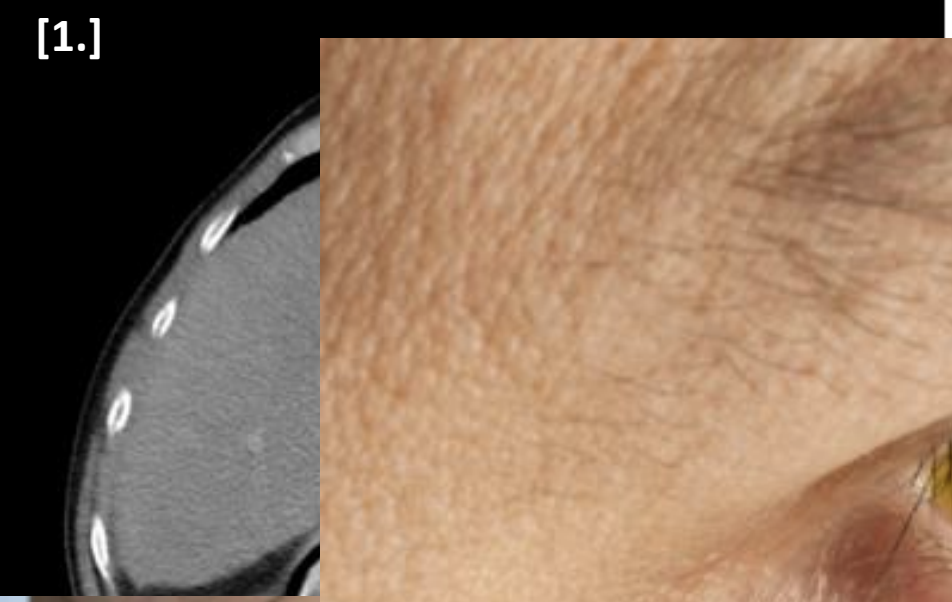
Introduction

1. Hepatocellular cancer (HCC) – some facts

- **HCC:** 6 most common malignancy; 3 leading cause of cancer related death (≈700,000 deaths/a)
- **Primary liver carcinomas:** 90% HCC, 10% Cholangiocellular Carcinoma
- **Treatment options:** hepatectomy, liver transplantation, ablation, Chemo, RT
- **Symptoms:** fluid in abdomen (ascites), jaundice (icterus), abdominal pain, loss of weight, fatigue, loss of energy
- **Risk factors:** 60-80% liver cirrhosis and hepatitis B/C, (aflatoxin, Paracetamol,...)
- **Problem:** high postoperative metastasis and recurrence rates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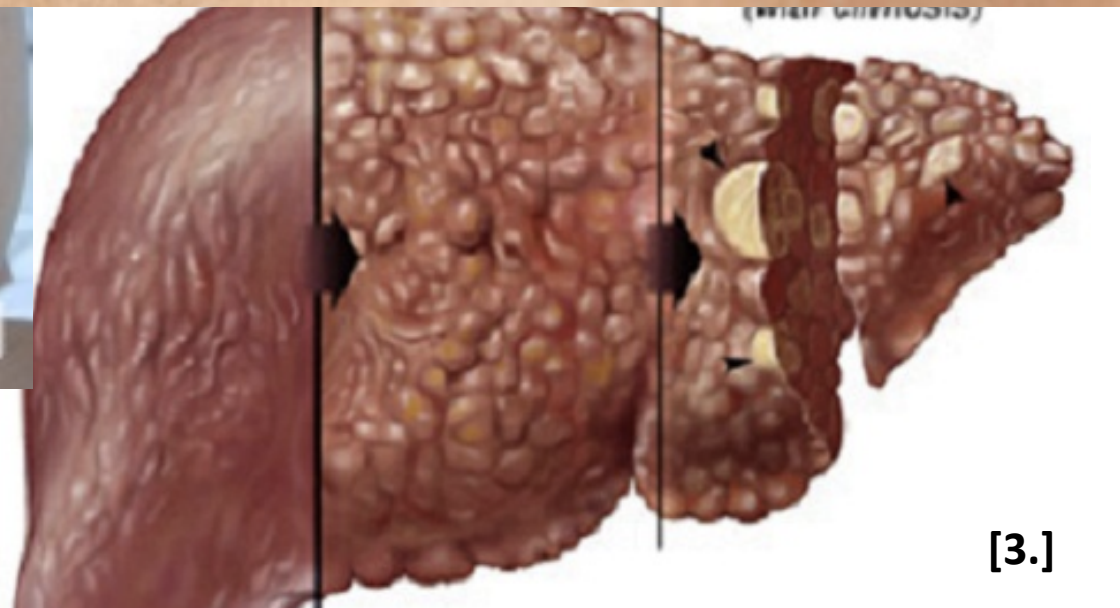
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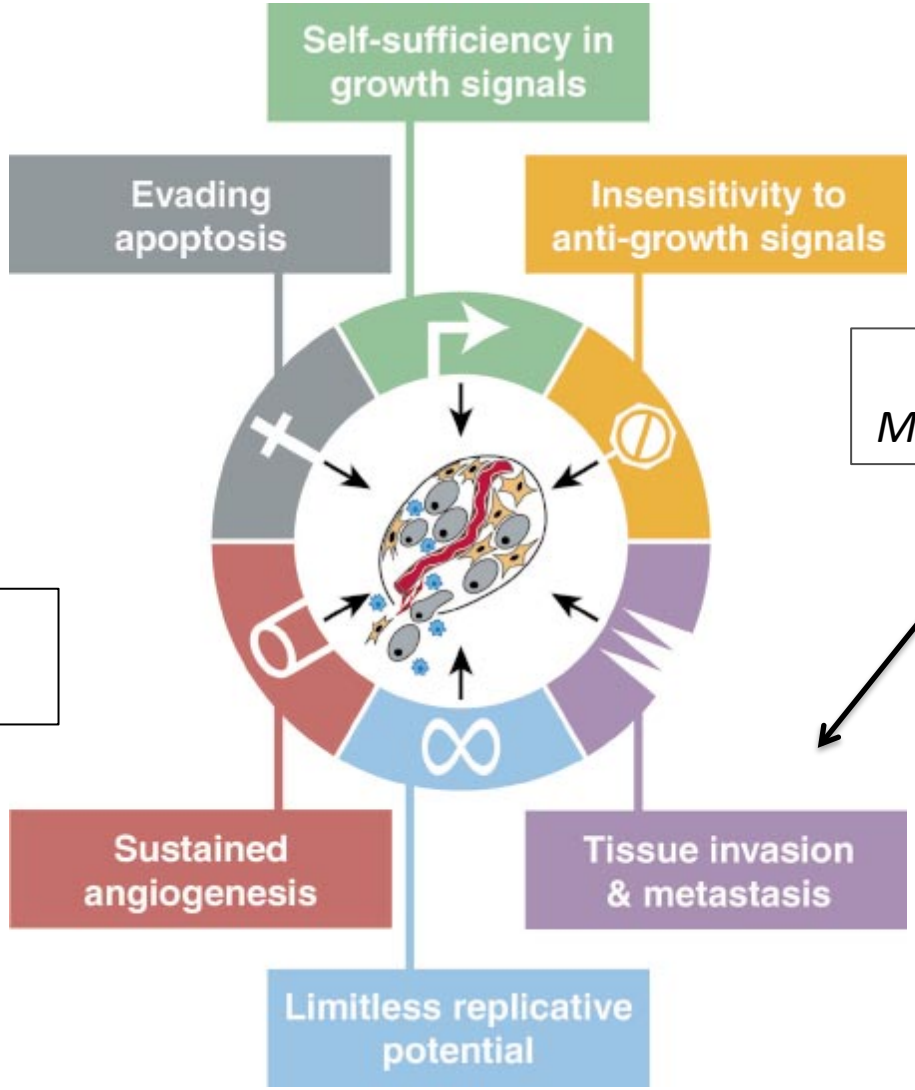
Hallmarks of Cancer



VEGF, VIII, CD34, CD31, MVD

EMT – *Epithelial to Mesenchymal Transition*

epithelial marker ↓
E-Cadherin

mesenchymal marker ↑
Vimentin

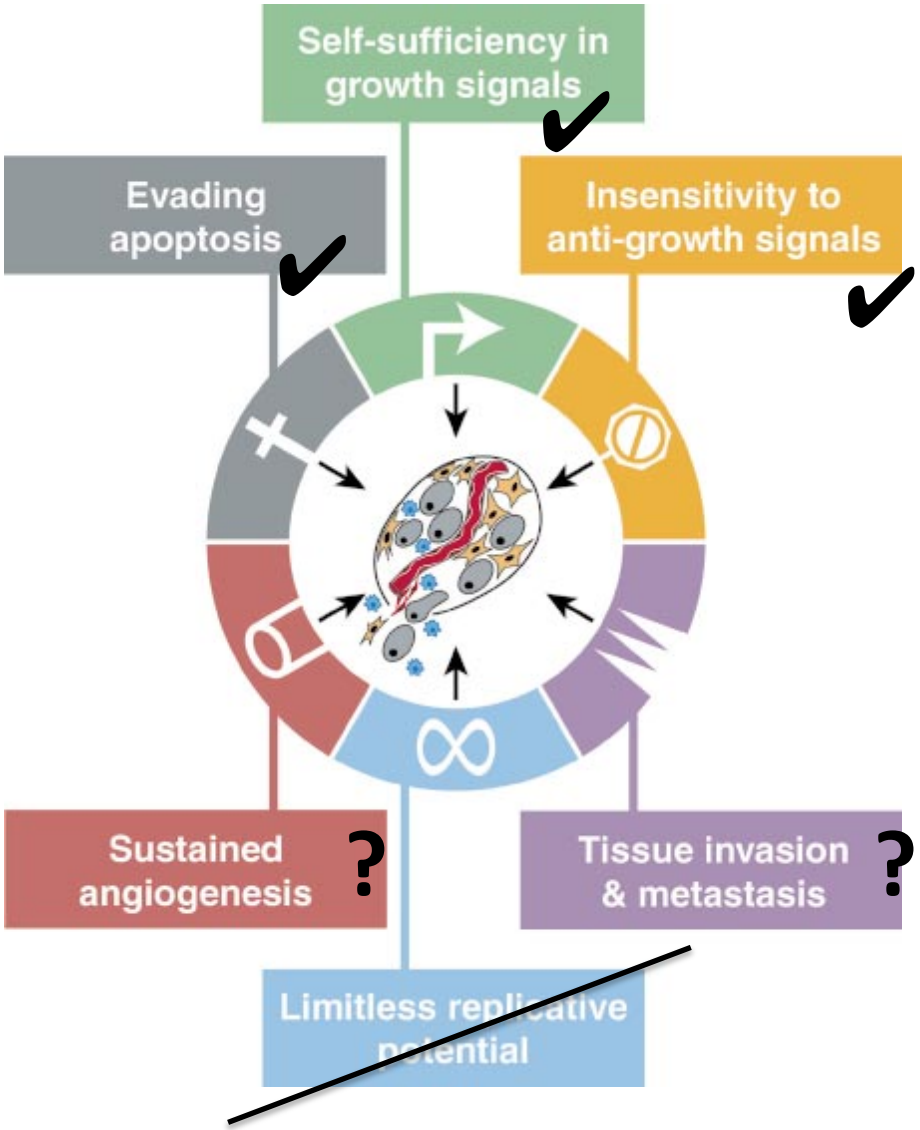
Hanahan D. et Weinberg RA. The Hallmarks of Cancer. Cell (2000), 100: 57-70

Mortalin = heat shock protein 75 (HSP75)

- Associated with tumor progression, bad prognosis, invasiveness and metastasis
- Highly expressed in several epithelial carcinomas (brain, lung, skin,...)
- Interacts with p53 suppressor gen -> inactivation in cancer cells *(Lu WJ. et al. 2011)*
- Modulates Ras-Raf-MAPK pathway -> cell proliferation *(Wadhwa R. et al. 2003)*
- Interaction with apoptosis *(Starenki D., et al. 2014)*

Objective: mortalin association with EMT and angiogenesis in HCC?

Mortalin (HSP75) and cancer



Hanahan D. et Weinberg RA. The Hallmarks of Cancer. Cell (2000), 100: 57-70

Methods (1)

Cell culture

- 5 human hepatoma derived tumor cell lines: **MHCC97-L, MHCC97-H, HCCLM3, Hep3B, HepG2**
- 1 normal liver cell line: **L02**

Tissue samples

- 96 HCC tissue specimens -> IHC
- 13 HCC tissue specimens + „paracarcinomatous tissues“ -> qPCR, western blotting
- 10 normal tissue liver samples (hepatic trauma)

Real-time quantitative PCR (qPCR)

- 6 cell lines, normal tissue samples, HCC tumor tissues + paracarcinomatous tissues
- **Protocol:** TRIzol -> cDNA synthesis kit (Invitrogen) -> Primer -> agarose gel
- **Primer:** Mortalin, Vimentin, GAPDH

Western blot

6 cell lines, normal tissue samples, HCC tumor tissues + paracarcinomatous tissues
Protocol: Protein Extraktion kit (Key Gen) -> SDS Page
Antibody: Mortalin, Vimentin, β-actin

Methods (2)

Immunohistochemistry (IHC)

1. **Mortalin** *(100 HCC+para tissues + 10 healthy controls)*
2. **Vimentin** *(100 HCC+para tissues)*
3. **CD 34** *(100 HCC+para tissues)*

- **Positive control:** breast cancer
- **Negative control:** PBS instead of primary antibody

Semiquantitative scoring (Mortalin, Vimentin)

1. 40x magnification: staining intensity (0-3)
2. 400x magnification: >5 fields (percentage of positive cells; 0, none, 1<10%, 2, 10%-50%, 3>50%
3. Total score: staining intensity x amount of positive cells (min.0, max. 9)
4. **High** immunoreactivity ≥4 total score, **low** immun. <4

Microvessel density (MVD), (CD34)

1. High power field (area of maximal angiogenesis)
2. Microvessels counted on 200x magnification

Methods (3)

Plasmid extraction and RNA interference -> Gen therapy

A)Protocol:

1. Small hairpin RNAs (shRNA) against Mortalin into GV115 vector
2. Plasmid -> bacteria for synthesis
3. Plasmid extraction (Plasmid Mini kit (Qiagen, China))

B) 3 Groups, 1 HHC human cell line (MHCC97H)

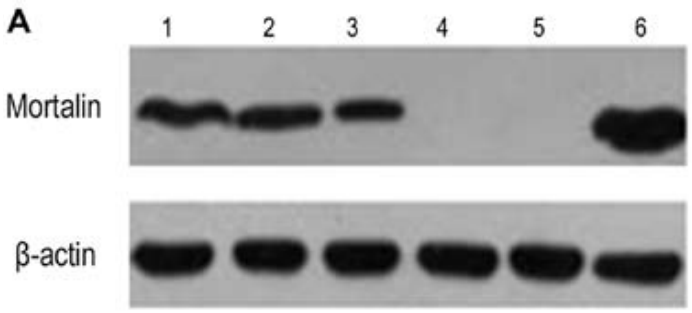
1. Blank group (no interference)
2. NC (negative control) -> transfected with NC shRNA
3. shRNA group -> transfected with Mortalin shRNA

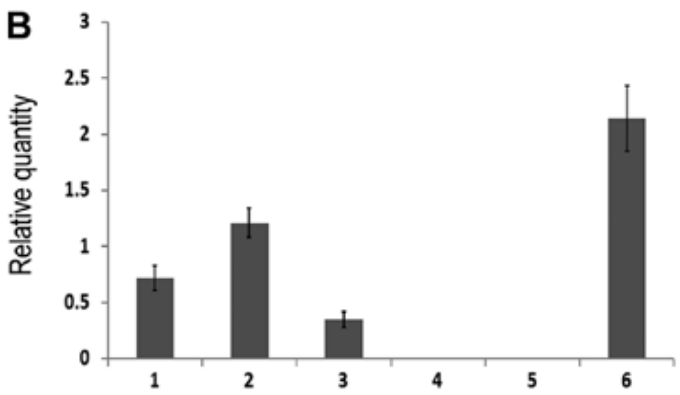
C) Harvesting of cells: 24, 48, 72, 96h

- MTT (MTT cell proliferation and cytotoxity assay kit)
  - 24 h after transfection, 96-well plates, MTT->ELISA
- low cytometry (annexin V/PI apoptosis kit)
  - 24h after transfection, centrifugation, annexin&propidium iodide (PI) -> analysis
- qPCR
- western blot

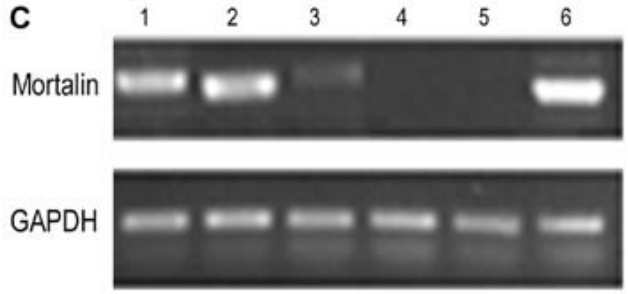
Results (1)

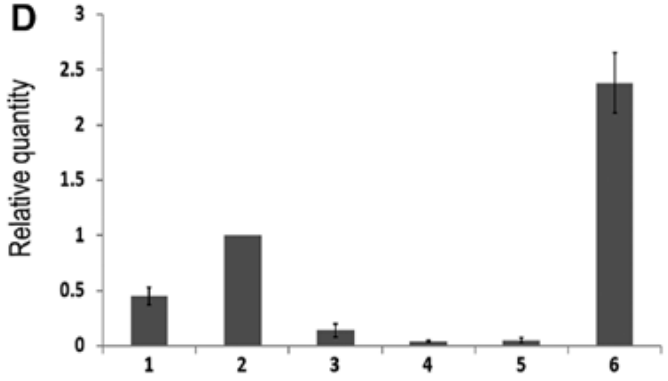
Western Blotting





qPCR





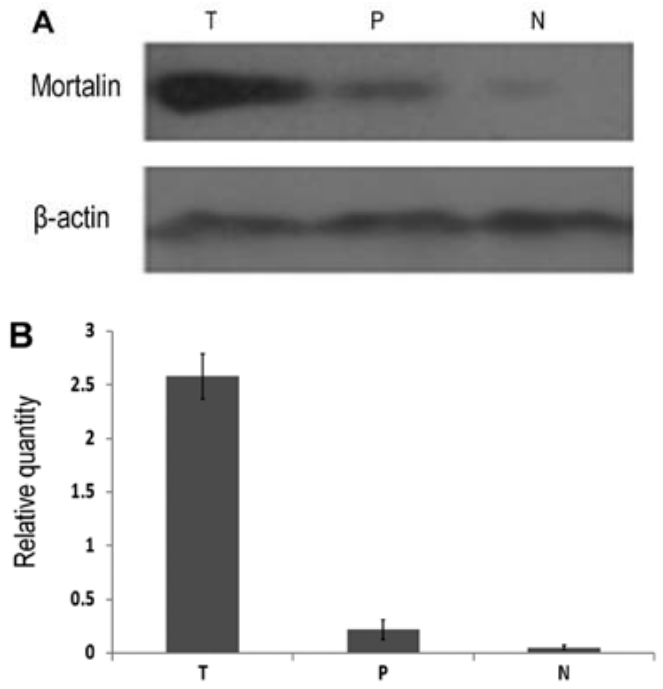
	Hep3B	MHCC97H	HepG2	L02	MHCC97L	HCCLM3
mRNA	0.45±0.08	1	0.14±0.06	0.04±0.01	0.05±0.02	2.38±0.27
Protein	0.72±0.11	1.21±0.13	0.35±0.07	0	0	2.14±0.29

HCC cell lines -> line with the highest metastatic potential (6), highest Mortalin expression **($p<0.05$)**

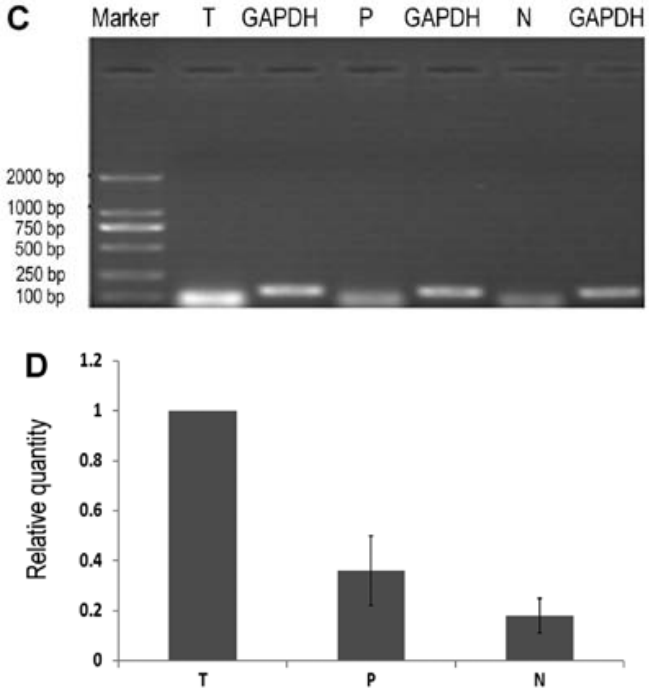
1. Hep3B
2. MHCC97H
3. HepG2
4. L02
5. MHCC97L
6. HCCLM3

Results (2)

Western Blotting



qPCR

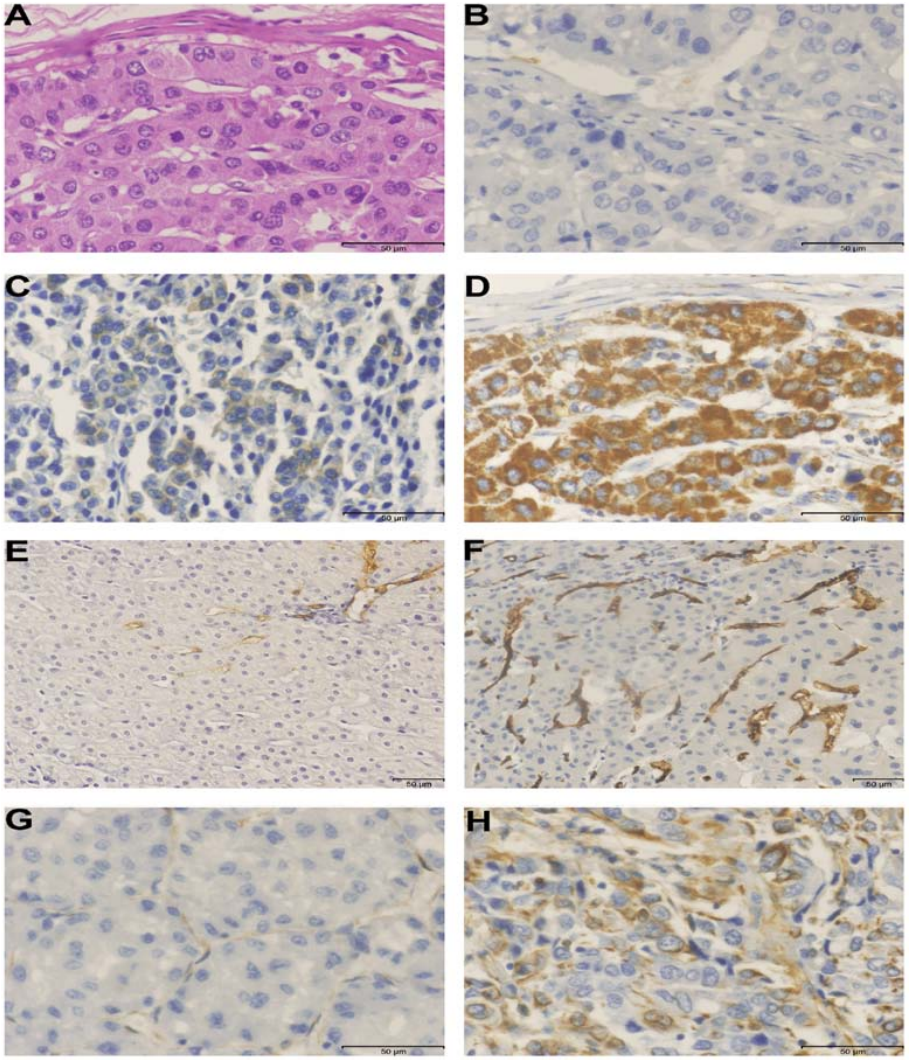


10 normal liver samples, 13 HCC samples and corresponding paracarcinomatous samples.

Expression level of Mortalin in HCC tumor tissues was significantly higher than in paracarcinomatous tissues and normal tissues **($p<0.05$)**

T... HCC tumor tissue
P... paracarcinomatous tissue
N... Normal tissue

Results (3)



A) HE staining, **B)** no expression of mortalin, **C)** low expression of mortalin, **D)** high expression of mortalin, **E)** low expression of MVD, **F)** high expression of MVD, **G)** low expression of Vimentin, **H)** high expression of Vimentin); A-D+G-H (x400), E-F (x200)

	Mortalin expression			
Liver tissue	Low	High	χ^2	P-value
HCC tumor tissue	23	77		
Paracarcinomatous tissue	81	19	67.388	<0.001
Normal tissue	9	1	16.669	<0.001

	Vimentin expression			
Mortalin expression	Low	High	r	P-value
Low	16	7	0.236	0.018
High	32	45		

Spearman's rank correlation

CD34 & Mortalin

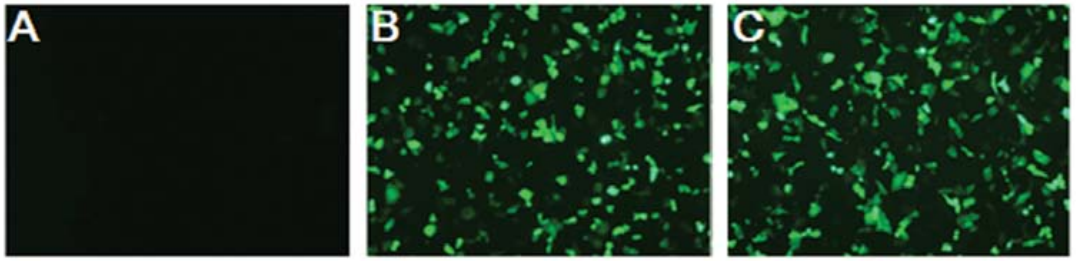
Tumors with high expression of Mortalin had a tendency to higher MVD than those with low expression of Mortalin (39.4±42.5 vs.29.7±16.9, **p=0.106**)

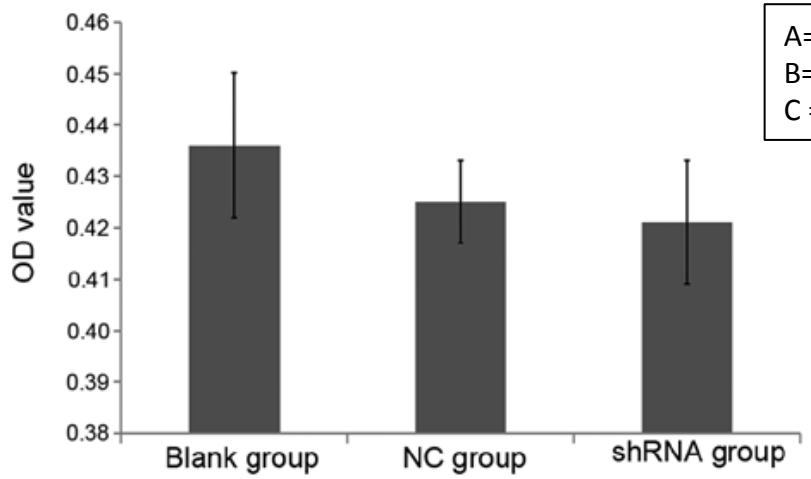
Results (4)

Characteristics	Case	Mortalin expression: Low	Mortalin expression: High	χ^2	P-value
Age (years)					
≤55	28	3	25	2.421	0.210
>55	72	20	52		
Gender					
Male	76	19	57	0.322	0.570
Female	24	4	20		
Tumor size (cm)					
≤5	38	5	33	3.352	0.067
>5	62	18	44		
Tumor nodules					
Single	68	18	50	0.898	0.343
Multiple	32	5	27		
Tumor capsula					
Complete	70	13	57	2.584	0.108
None	30	10	20		
AFP (ng/ml)					
≤400	31	7	24	0.004	0.947
>400	69	16	53		
$ICGR_{15}$ (%)					
≤10	79	17	62	1.697	0.193
>10	21	6	15		
HBsAg					
Positive	84	20	64	0.014	0.907
Negative	16	3	13		
Liver cirrhosis					
Present	87	21	66	0.004	0.951
Absent	11	2	9		
Child-Pugh grade					
A	79	15	64	3.420	0.064
B	21	8	13		
Edmondson grade					
I-II	67	20	47	4.272	0.039
III-IV	33	3	30		
TNM stage					
I-II	64	21	43	8.188	0.004
III-IV	36	2	34		
Invasion and metastasis					
Absent	61	19	42	4.742	0.029
Present	39	4	35		

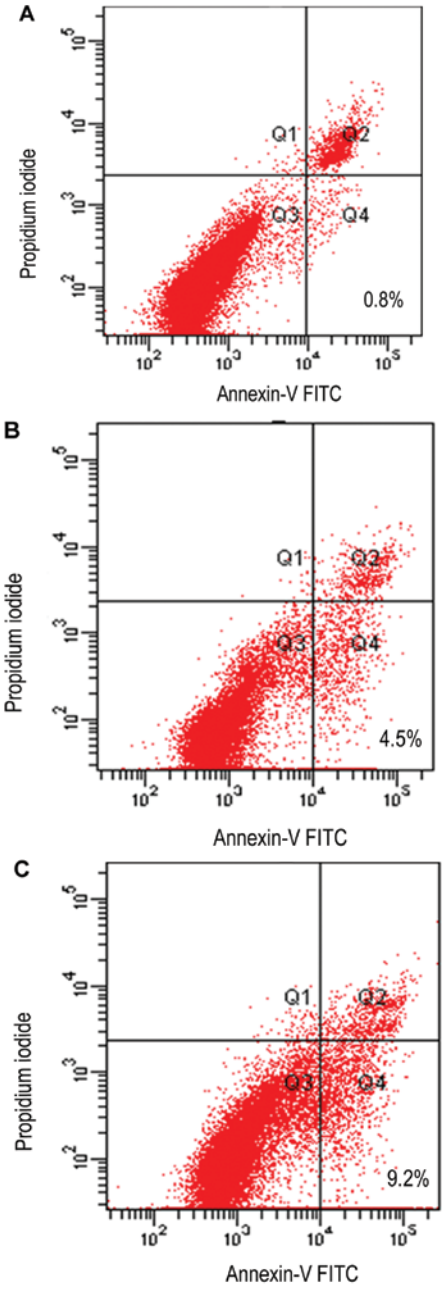
$ICGR_{15}$, indocyanine green retention rate at 15 min.

Results (5)

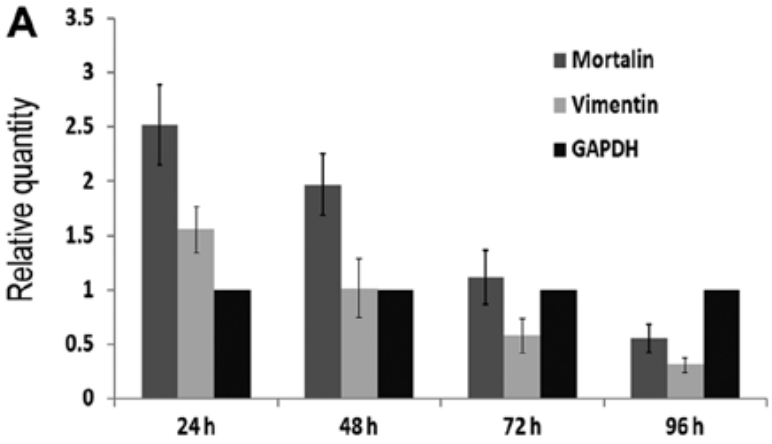


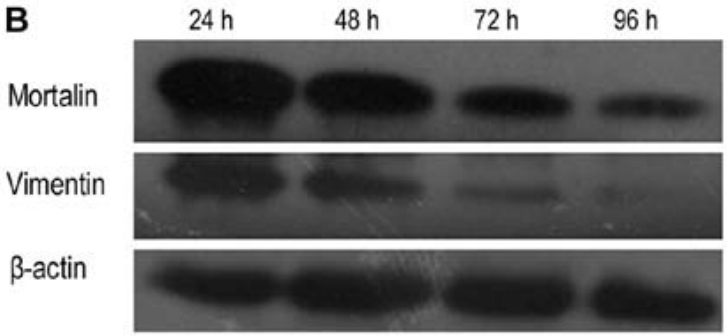


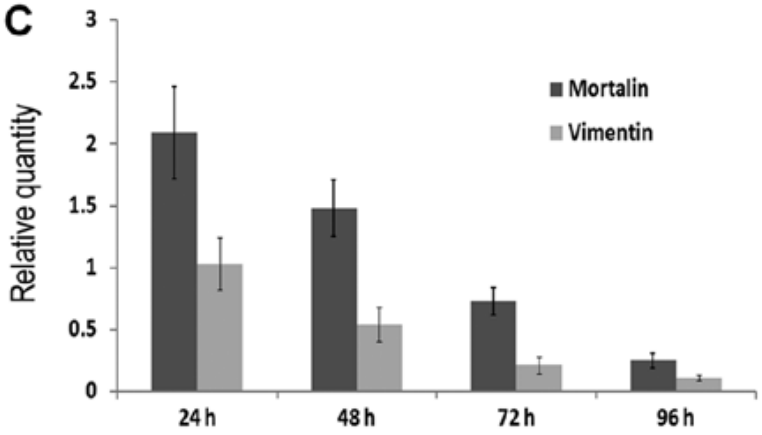
A=blank group
B=NC group
C =shRNA group



- **GFP fluorescence** -> successful transfection-> **MHCC97H cells**
- **MTT&flow cytometry:** 24h after transfection; **p>0.05**
  transfection did not cause severe cell damage!







Results (6)

A)Mortalin / Vimentin mRNA

- **24h:** 2.52±0.37 / 1.56±0.21
- **48h:** 1.97±0.28 / 1.02±0.27
- **72h:** 1.12±0.25 / 0.58±0.16
- **96h:** 0.55±0.13 / 0.31±0.07
- *(no change in NC and blank group!)* **p<0.05**

B) Mortalin / Vimentin protein

- **24h:** 2.09±0.37 / 1.03±0.21
- **48h:** 1.48±0.23 / 0.54±0.14
- **72h:** 0.73±0.11 / 0.21±0.07
- **96h:** 0.25±0.06 / 0.11±0.02

C)Mortalin & Vimentin expression

- Decreased expression of Mortalin was accompanied by reduction of Vimentin expression
- **Inhibition of Mortalin expression could decrease Vimentin expression and could have suppressive effect on EMT!**

Conclusion

- Mortalin was higher expressed in HCC tumor specimens
- Mortalin significantly correlation with Vimentin (EMT)
- High Mortalin expression was not related to higher MVD
- Mortalin correlated with level of metastasis/invasiveness (TNM, Edmondson grade)
- Overexpression of Mortalin could possess metastatic - inducing capabilities
- Mortalin shRNA transfection led to decreased Mortalin levels and was accompanied by a reduction of Vimentin

- **Mortalin expression could promote EMT**
- **Mortalin had no influence on angiogenesis**

shRNA transfection (Mortalin knockdown) -> potential clinical application to decrease tumor metastasis and recurrence after curative surgery by inhibiting EMT!!!

Sources

Figures

1. HCC early stage: radiofrequence ablation; http://www.cancernews.com/data/Article/504.asp
2. Advanced stage HCC; http://liveratlas.org/case/88/
3. Pathogenesis of HCC; http://cancerssymptoms.org/liver-cancer-2
4. Livercirrhosis; http://www.medicoconsult.de/wiki/Leberzirrhose_in_Bildern
5. Icterus; http://www.praxisvita.de/wenn-die-leber-um-hilfe-schreit

Papers

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- **Lu WJ., et al.** Mortalin-53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy. Cell Death and Differentiation (2011); 18:1046-56
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- **Starenki D, Hong SK, Lloyd RV, Park JI.** Mortalin (GRP75/HSPA9) upregulation promotes survival and proliferation of medullary thyroid carcinoma cells. Oncogene. 2014 Dec 1. doi: 10.1038/onc.2014.392. [Epub ahead of print]