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

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JC Current Topics in Applied Immunology WS 2014/15, Wien 19.1.15
1. Hepatocellular cancer (HCC) – some facts

- **HCC**: 6 most common malignancy; 3 leading cause of cancer related death
  \((\approx 700,000\) 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
Normal cells require mitogenic growth signals (GS) because their proliferation is dependent on growth stimulation from their normal tissue microenvironment. However, tumor cells have acquired the ability to generate their own growth signals, thereby reducing their dependence on exogenous growth stimulation. The consequence is that tumor cells generate many of their own mitogenic factors and a proper substratum for their interaction, allowing them to proliferate only when supplied with appropriate diffusible signals into the cytoplasm that influence cell behavior.

We suggest that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through variegated mechanistic strategies. We describe each capability in turn below, illustrate with a few examples its functional importance, and indicate various mechanistic strategies by which it is acquired in human cancers. We describe each capability in turn below, illustrating with a few examples its functional importance, and indicating various mechanistic strategies by which it is acquired in human cancers.

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

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 cytotoxicity 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)

HCC cell lines -> line with the highest metastatic potential (6), highest Mortalin expression (p<0.05)
Results (2)

Western Blotting

qPCR

A

B

C

D

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

10 normal liver samples, 13 HCC samples and corresponding paracarcinomatous samples.
Results (3)

<table>
<thead>
<tr>
<th>Liver tissue</th>
<th>Mortalin expression</th>
<th>( \chi^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC tumor tissue</td>
<td>23</td>
<td>77</td>
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<tr>
<td>Paracarcinomatous tissue</td>
<td>81</td>
<td>19</td>
<td>67.388</td>
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<td>Normal tissue</td>
<td>9</td>
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<td>16.669</td>
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<table>
<thead>
<tr>
<th>Mortalin expression</th>
<th>Vimentin expression</th>
<th>r</th>
<th>P-value</th>
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<tr>
<td>Low</td>
<td>Low</td>
<td>16</td>
<td>7</td>
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<tr>
<td>Low</td>
<td>High</td>
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<td>45</td>
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<tr>
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<tr>
<td>High</td>
<td>High</td>
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</table>

*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, \( p=0.106 \))
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case</th>
<th>Low</th>
<th>High</th>
<th>$\chi^2$</th>
<th>P-value</th>
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<td>Age (years)</td>
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<td>ICGR$_{15}$ (%)</td>
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</table>

ICGR$_{15}$, indocyanine green retention rate at 15 min.
Results (5)

- **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!!!
Figures
1. HCC early stage: radiofrequency ablation; http://www.cancernews.com/data/Article/504.asp
2. Advanced stage HCC; http://liveratlas.org/case/88/
4. Livercirrhosis; http://www.medicoconsult.de/wiki/Leberzirrhose_in_Bildern
5. Icterus; http://www.praxisvita.de/wenn-die-leber-um-hilfe-schreit

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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]