**Low dose paclitaxel reduces S100A4 nuclear import to inhibit invasion and hematogenous metastasis of cholangiocarcinoma**

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**Supplemental Materials**

*Cell apoptosis*. Since in some treatments with rapid cytotoxicity, reduction in mitochondrial activity does not affect cell survival and therefore, analysis of cell viability by MTS might underestimate the number of dead cells, c*ell apoptosis* was assessed by immunofluorescence for cleaved caspase 3 (CC3, 1:500, rabbit, Cell Signaling) and then developed. Results were expressed as percentage of cells with positive cytoplasmic staining for CC3 on the total number of cells, in 20 random fields taken at 200x magnification, by two independent observers (MC, LS) as previously described (9).

*Cell migration (wound healing) assay.* CCA cell lines were seeded in a 6-well plate, grown until confluence, and then starved for 24h. Each cell monolayer was scratched three times with a sterile p200 tip, and three micrographs were taken at t=0h for each wound. Then, on the same scratched area, micrographs were taken again at 24h and 72h to measure the distance between the wound margins using LuciaG 5.0 software (Nikon), and expressed by normalizing each time point to t=0h (9,13).

*Membrane-type 1 (MT1) matrix metalloproteinase (MMP) expression*. To assess the membrane expression levels of MT1-MMP, immunofluorescence staining was analyzed using the LuciaG 5.0 software (Nikon). After selecting a region of interest between two adjacent tumor cells, the relative labeling intensity was evaluated on either red (MT1-MMP, red line) or blue (DAPI, nuclei, blue line) channels, and the higher peak of red fluorescence was measured (Supplemental Fig. 4B).

*Activity of Rho-A, Rac-1 and Cdc-42 GTPase.* CCA cells were seeded and cultured for three days to reach at least the 70% of confluence to assess Rho-A and Rac-1 GTP levels, and the 20-30% of confluence for Cdc-42 GTP levels. Then, confluent cells were exposed to PTX, and proteins were extracted to perform the G-LISA assay according to the supplier (Cytoskeleton Inc.).

*MMP-9 secretion.* Fifty-thousand cells were seeded on a 24-well plate and then treated with PTX. Supernatant was harvested, stored at -80°C, and ELISA performed to quantify both the pro and the active MMP-9 forms (RayBiotech).

*Gelatin zymography assay.* One hundred µg of total protein lysate was loaded on each well of a 10% Tris-Glycine + 0.1% Gelatin gel (LifeTechnologies) and proteins were run for 90mins using a Tris-Glycine Buffer. Gels were then incubated with renaturing buffer for 30min at room temperature (LifeTechnologies), and incubated in developing buffer (LifeTechnologies) for 4h at 37°C. Finally, gels were stained for 30min with Coomassie Brilliant blue (Bio-Rad) and pictures were taken using the Gel Logic 100 Imaging System (Kodak).

*Immunohistochemistry for S100A4, p-Hist3 and CC3 in SCID mice spleen specimens.* The sections derived from spleen were immunostained for S100A4 (1:400), p-Hist3 (1:50, rabbit, Cell Signaling), and CC3 (1:300), developed with DAB and H2O2 0.01% and counterstained with Gill’s Haematoxylin N°2 (Sigma). All antibodies were diluted in PBS 1M, and supplemented with 1% BSA and 0.05% Tween20 (Sigma). Results were expressed as percentage of positive cells/field at 200x in 10 randomly taken fields, as previously performed (9). Positive staining for S100A4 and p-Hist3 was considered as expression of intense brown staining in the nucleus, whereas positivity for CC3 was represented by granular brown staining in the cytoplasm of EGI-1 cells.