SUPPLEMENTARY ON-LINE MATERIAL

METHODS

Patients and tissue samples. Histological specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin. All specimens were reviewed by pathologists (AS, AF, MR, CM) to confirm the diagnosis of CCA and to allow their correct categorization according to the current TNM criteria. Clinical outcomes were registered by examining local mortality records and by phone interviews, when the information was not on record at the medical centers.

Immunohistochemical analysis of human CCA specimens. Histological sections were immunostained using a rabbit polyclonal antibody anti-S100A4 (Dako, Milan) or a mouse monoclonal antibody anti-cytokeratin-19 (K19) (clone RCK108, DAKO, Milan). After deparaffinisation, sections were hydrated in alcohol and endogenous peroxidase activity was quenched for 30 min in methilic alcohol with 10% of hydrogen peroxide (10%). Antigen retrieval was performed by heating slides for 20 min in 10mM cytrate buffer, pH6.0 in a steamer. Sections were incubated overnight at 4°C with anti-S100A4 primary antibodies (1:400) or 1h at room temperature for anti-K19 primary antibody (1:50); liver sections were rinsed with 0.05% Tween 20 in PBS and incubated for 30 min at room temperature with the appropriate Dako EnVision anti-Rabbit or anti-Mouse (Dako, Milan). Specimens were developed using 3,3-diaminobenzedine tetrahydrochloride 0.04mg/ml (DAB, Sigma, Milan) and H_2O_2 0.01% and counterstained with Gill's Hematoxilin N°2 (Sigma, Milan). All the antibodies were diluted in phosphate buffered saline (PBS, Sigma, Milan) 1M supplemented with 5% normal human serum type 0 and 0.05% Tween20 (Sigma, Milan). Nuclear expression of S100A4 was assessed by computer-assisted morphometry using LuciaG 5.0 software (Nikon, Milan). Positive nuclei in cholangiocytes lining neoplastic ducts were counted in 10 micrographs randomly taken at 200x magnification for each sample, and expressed as percentage of the overall number of nuclei in neoplastic ducts. Tissue samples were considered negative when no S100A4 nuclear expression on neoplastic ducts could be observed

throughout the histological section. Immunohistochemical expression of K19 on neoplastic ducts in CCA tissue sections was staged by two independent observers (MC, LS) and divided into 3 groups, as negative, weakly positive (<30% K19-positive ducts), and strongly positive (≥30 K19-positive ducts).

Survival and multivariate analysis. As S100A4 was distributed highly asymmetrically with a preponderance of subjects that had no detectable expression of nuclear S100A4, Kaplan-Meier analysis and multivariate analysis were performed either by considering S100A4 as a continuous variable or by categorizing S100A4 nuclear expression into 3 levels, negative (S100A4=0), weakly positive (S100A4<30%) and strongly positive groups (S100A4≥30). These values were chosen on the basis of the median percentage (30%) of S100A4 expression calculated in patients with detectable nuclear expression. Multivariate Cox regression was used to examine the association between S100A4 nuclear expression and survival while adjusting for age, sex, intra or extra-hepatic localisation, involvement of surgical resection margins, grade of tumour differentiation, and TNM classification parameters including tumor size and lymph node involvement.

Out of the 86 patients undergoing resection and surviving more than 30 days after surgery, originally considered in the Kaplan-Meyer and Cox regression analysis, we have performed a new analysis in the 67 subjects (78%), for which records on metastasis were available. This subset of patients was well representative of the entire series in terms of clinical features, including outcome, as well as of expression levels of nuclear S100A4 (Table S2). However, the exact timing in which metastasis developed could not be established with accuracy from our data-base since follow-up imaging studies were not performed in the three centres according to the same protocol. Therefore, to overcome the limitations related to the interval censored data, instead of using Kaplan-Meier method as originally performed when analyzing death as outcome, we have generated a survival curve using a Nonparametric Maximum Likelihood Estimator (NPMLE) and a Generalized log-Rank Test, as reported by Sun et al[10]. The macro allowing this analysis is published by the SAS

institute and is currently used in SAS. All analyses were performed using SAS Version 9.2 (SAS, Cary, NC).

CCA cell lines and detection of S100A4 and K19 by immunofluorescence. EGI-1 and TFK-1 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Both are extra-hepatic CCA cell lines obtained from explanted livers of male patients[11,12]. EGI-1 and TFK-1 were cultured in RPMI 1640 (PAA, Milan) supplemented with 10% FBS (PAA, Milan) and 1% Penicillin/Streptomycin (Invitrogen, Milan), in 5% CO_2 humified atmosphere at 37°C. In cultured cells, S100A4 immunoreactivity was detected by immunofluorescence. Briefly, after fixation with 4% paraphormaldehyde (Carlo Erba, Milan), cells were incubated overnight at 4°C with rabbit anti-S100A4 (1:400) or with mouse anti-K19 (1:100) and after washing with PBS, cells were supplemented with 0.05% Tween 20, and then incubated 30 min at room temperature with goat anti-rabbit or anti-mouse Alexa fluor 499 (1:200, Invitrogen, Milan) and mounted with Vectastain + DAPI (Vector Laboratories).

Western blot analysis. Cytoplasmic and nuclear expression of S100A4 was also evaluated by western blot (WB) on cytoplasmic and nuclear cell fractions. Methological details are given in the supplemantary material section. (Ne-Per, Thermo Scientific, Milan) according to supplier instructions. Protein concentration was measured with Bradford method with the Epoch microplate reader (Biotek, Winooski, VT, USA). Equal amounts of lysate were applied to a 4-12% NuPAGE® Novex Bis-Tris gel (Invitrogen, Milan) and electrophoresed. Proteins were transferred to nitrocellulose membrane (Invitrogen, Milan). Membranes were blocked with 5% non-fat dry milk (Sigma, Milan) in TBS supplemented 0.1% Tween-20 (TBST) for 1h and then incubated overnight at 4°C with rabbit-anti-S100A4 (1:1000, Dako, Milan). As reference proteins, we used anti-Actin (1:2000, Sigma, Milan) for cytoplasmic extracts and anti-Hystone H3 (1:2000, Sigma, Milan) for nuclear extracts. Nitrocellulose membranes were washed three times with TBST and then incubated with goat-anti-mouse (1:2000, Sigma, Milan) or with goat-anti-rabbit (1:2000, Bio-Rad, Milan)

horseradish peroxidase-conjugated secondary antibodies for 1h. Proteins were visualized by enhanced chemiluminescence (SuperSignal West Pico chemiluminescent, Thermo Scientific, Milan).

Xenotransplantation in SCID mice. Mice were anesthetized and the spleen was carefully exteriorized through a peritoneal incision by the use of forceps. Five-hundred thousand cells in a total volume of 100 µl were injected and soon afterwards the spleen was replaced in the abdominal cavity and the abdominal muscle and skin were sutured. Procedures involving animals and their care were conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, December 12, 1987). *In vivo* bioluminescence imaging was performed using the IVIS™ Imaging System (Xenogen Corporation, Almeda, CA). Ten minutes before imaging, animals were anesthetized and administered intra-peritoneum (i.p.) with D-luciferin (Biosynth AG, Staad, Switzerland; 150 mg/kg). Metastatic spread was systematically checked at weekly intervals by *in vivo* bioluminescence imaging using the Living Image® software (Xenogen), and considered as positive when a new signal at distance from the spleen could be detected. When metastatic spread was detected, mice were sacrificed to confirm at autopsy the presence of new tumor masses grown at distance from the site of injection. In addition, 5 serially cut sections with a 70µm-interval taken from the liver, lung, ovaries and kidneys of sacrificed mice were stained by H&E to evaluate the presence of micrometastases.

Immunohistochemical analysis of SCID mice specimens. Sections were immunostained using a rabbit polyclonal antibody anti-MMP-2 (AbCAM, Milan), a rabbit polyclonal anti-MMP-9 (Cell Signaling, Milan), and a mouse monoclonal antibody anti-K19 (clone RCK108, DAKO, Milan). After deparaffinisation, sections were hydrated in alcohol and endogenous peroxidase activity was quenched for 30 min in methilic alcohol with 10% of hydrogen peroxide. Antigen retrieval was performed by incubating slides for 5min in pepsin solution (Invitrogen, Milan) at 37°C for MMP-2 or by heating slides for 20min in 10mM cytrate buffer, pH=6.0 in a steamer for MMP-9. To avoid

aspecific binding, specimens were preincubated for 30min at room temperature with PBS supplemented with 5% BSA and 0,05% Tween 20, then sections were incubated overnight at 4°C with anti-MMP-2 (1:150) or anti-MMP-9 (1:100). Sections were rinsed with 0.05% Tween 20 in PBS 1M and incubated for 30min at room temperature with Dako EnVision anti-Rabbit, developed using DAB and H_2O_2 0.01% and counterstained with Gill's Hematoxilin N°2 (Sigma, Milan). All the antibodies were diluted PBS 1M supplemented with 1% BSA and 0.05% Tween20 (Sigma, Milan).

Lentiviral silencing of nuclear S100A4 in EGI-1 cells. Vector stocks were generated by a transient three-plasmid vector-packaging system in 293T cells as detailed elsewhere[15]. Briefly, 293T packaging cells were seeded in T75 flasks at 5 x 10^6 cells/flask, and transfected overnight according to a calcium-phosphate protocol using 12 µg of a shRNA or reporter gene plasmid, 6 µg of the packaging construct p8.74, and 1 µg of a VSV-G expression plasmid. Eighteen hours after transfection, culture medium was replaced with fresh DMEM; 24h later the viral vector-containing supernatants were harvested, passed through 0.45 mm filters, concentrated by ultracentrifugation and stored at –80°C until use. For transduction of EGI-1 cells by shRNA lentiviral vector, 1 µg of p24 equivalent of each concentrated vector-containing supernatant was layered over target cells that had been seeded into 25 cm^2 culture flasks at 7x10^5 cells. After 6-9h at 37°C, the supernatant was replaced with complete medium. Seventy-two hours after transduction, the cells silenced for S100A4 were selected by their resistance to puromycine (5mg/ml). Effectiveness of S100A4 silencing was then evaluated by WB on nuclear and cytoplasmic cell fractions, as previously described. In EGI-1 cells silenced for S100A4, immunocytochemistry for K19 was also detected and compared to parental EGI-1 cells to see if down-regulation of S100A4 was associated with alteration of epithelial markers.

Cell migration (Wound healing) assay. Cells were seeded in a 6-well plate, grown until confluence, and then starved for 24 h to synchronize cell proliferation. After starvation, each cell monolayer

was scraped three times with a sterile p200 tip, and in each wound three micrographs were taken at t = 0 h[16]. On the same area of the scratch, micrographs were then taken at 24 h and 72 h to measure the distance of the scratch edge using LuciaG 5.0 software (Nikon, Milan), and each time point was normalized to t=0h.

Cell invasion (Boyden chamber) assay. CCA cells were seeded over a reconstituted basement membrane (Matrigel™) coated on filter in a Boyden chamber[17]. Briefly, cells were resuspended in serum-free medium. Polyvinylpyrolidone-free polycarbonate membrane 8µm-pore filters (Transwell, Costar, Milan) coated with 50µg/ml matrigel was placed in a Boyden microchamber. The lower compartment was filled up with culture serum-free medium to evaluate cell invasiveness in lack of any chemotactic stimulus. Six wells were used for each experimental condition, whereby $2.5x10^6$ cells were added to the upper compartment of the chamber and incubated for 48h at 37°C in a 5% CO_2/95% air atmosphere. To evaluate the number of fully migrated cells, the cells on the upper surface were removed with a cotton swab and the lower surface of the transwell filter was stained with Diff-Quick Staining Set (Medion Diagnostics, Milan) and then micrographs of 20 random fields were taken on each filter to count the number of clearly discernible nuclei.

Cell proliferation assay. Cells maintained in $25cm^2$ tissue culture flasks in medium enriched with 10% FBS at 37°C in a humidified, 5% CO_2 atmosphere, were starved for 24 h and passaged and then cultured in a 96-multiwell plate (5000 cells/well) with quiescent medium, without FBS[18]. After 24h, cells were supplemented with growth medium supplemented with 10% FBS (Invitrogen, Milan). Proliferation was assessed by the CellTiter 96 AQueous One Solution (Promega Italia, Milan, Italy), which exploits the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) (MTS) -2*H*-tetrazolium compound colorimetric bioreduction by the cells.

Detection of cleaved caspase-3 by immunofluorescence. Human CCA cell lines were cultured and fixed as above outlined. Cells were incubated overnight at 4°C with rabbit anti-cleaved caspase-3 (1:200) and after washing with PBS supplemented with 0.05% Tween 20, and then incubated 30min

at room temperature with goat anti-rabbit Alexa fluor 499 (1:200, Invitrogen, Milan) and mounted with Vectastain + DAPI (Vector Laboratories). Results were expressed as percentage of positive cells on total cultured cells in 20 non-overlapping random fields taken at 200x. Pictures were analyzed using LuciaG5.0 software (Nikon, Florence).

MMP-2 and MMP-9 ELISA assay. Fifty thousand cells were seeded onto 25-wells plate and after 24h of incubation surnatant medium was harvested for ELISA analysis. Secretion of MMPs was evaluated using MMP-2 and MMP-9 specific ELISA kit (Raybiotech, Milan) as indicated by the supplier. For each set of experiments a standard curve was generated for both MMP-2 and MMP-9 to allow quantification.

Statistical analysis. Nonparametric correlation between nuclear S100A4 and K19 expression by neoplastic ducts in histological serial sections of CCA specimens was calculated by Spearman's rho test. Results of animal and *in vitro* experiments are shown as mean ± standard deviation. Statistical comparisons were made using Student's t-tests or the Wilcoxon-Mann-Whitney two-sample rank-sum test, where appropriate. In the latter, the p-value was obtained from the exact permutation null distribution. The statistical analysis was performed using SAS Version 9.2 (SAS, Cary, NC); p values <0.05 were considered as significant.

RESULTS

Down-regulation of S100A4 expression in EGI-1 cells results in a significant reduction in cell motility, invasion and MMP-9 secretion, without affecting cell proliferation and apoptosis.

Cell proliferation. Cell proliferation was investigated in EGI-1 and in sh8 and sh9 clones, using the MTS assay. As shown in Figure S1 (see supplementary on-line materials), silencing of nuclear S100A4 expression did not result in a significant reduction in the proliferative activity of EGI-1 cells, as compared to wild type and to scrambled EGI-1.

Cell apoptosis. Apoptosis was assessed by morphometric count of the percentage of cleaved Caspase-3 positive cells by immunocytochemistry in EGI-1, shRNA, sh8 and sh9 cultured cells. Expression of cleaved Caspase-3 was not different in the different cell groups (0.06±0.09 in EGI-1, 0.03±0.06 in shRNA, 0.12±0.21 in sh8, 0.59±0.55 in sh9, p=ns in all cases).

Legend to Supplementary on-line Figures

Figure S1. Hazard functions for death and metastasis with Weibull model. The estimated hazard function for death (A) and metastasis (B) are shown. For death, the hazard function was increased with a rate decreasing over time (A), whereas for metastasis, the hazard is very high at the beginning but it rapidly decreases over time (B).

Figure S2. Immunocytochemical expression of K19 in human CCA cell lines is not altered by lentiviral silencing of S100A4. K19 expression (green) was similar in EGI-1 (A), sh8-EGI-1 (B) and sh9-EGI-1 (C) cells as well as in TFK-1 (D) cell line. Nuclei were counterstained with DAPI (blue). Original magnification: 200x.

Figure S3. Functional effects of S100A4 silencing in EGI-1 cells as compared to TFK-1 cells on cell proliferation. Using MTS assay after a 24h incubation with growth medium, no difference in the proliferative activity was found among silenced, scramble, EGI-1 and TFK-1 cells.

Figure S4. MMP-2 and MMP-9 expression in EGI-1 cells and functional effects of S100A4 silencing in EGI-1 cells on MMP-9 secretion. Tissue sections of liver metastases derived from xenotransplantation of EGI-1 cells in SCID mice immunostained for MMP-2 (A) and MMP-9 (B) showed that MMP-9, but MMP-2, was strongly expressed by metastasizing CCA cells. Immunoperoxidase technique, original magnification: 200x. In the supernatant of cultured cells (n=4), MMP-9 secretion by EGI-1 cells assessed by ELISA was significantly reduced after lenitiviral silencing of S100A4, as observed in sh8 and sh9 clones ($p<0.001$); in TFK-1 cells, MMP-9 secretion is negligible (C).