Life Sciences 277 (2021) 119494

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

A tumorigenic cell line derived from a hamster cholangiocarcinoma associated with *Opisthorchis felineus* liver fluke infection

Viatcheslav A. Mordvinov [a,*], Galina A. Minkova [a], Anna V. Kovner [a], Dmitriy V. Ponomarev [a], Maria N. Lvova [a], Oxana Zaparina [a], Svetlana A. Romanenko [b], Alexander G. Shilov [a], Maria Y. Pakharukova [a,c]

[a] Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences (ICG SB RAS), 10 Lavrentiev Ave., Novosibirsk 630090, Russia
[b] Institute of Molecular and Cellular Biology, Siberian Branch of Russian Academy of Sciences, 8/2 Lavrentiev Ave., Novosibirsk 630090, Russia
[c] Department of Natural Sciences, Novosibirsk State University, 2 Pirogova Str., Novosibirsk 630090, Russia

ARTICLE INFO

Keywords:
Opisthorchis felineus
Hamster
Cholangiocarcinoma
Cell line
Experimental model

ABSTRACT

Aims: The food-born trematode *Opisthorchis felineus* colonizes bile ducts of the liver of fish-eating mammals including humans. There is growing evidence that this liver fluke is a risk factor for cholangiocarcinoma (CCA). Cancer cell lines are necessary for drug screening and for identifying protein markers of CCA. The aim was to establish a cell line derived from cholangiocarcinoma associated with opisthorchiasis felinea.

Main methods: Allotransplantation, immunohistochemistry, karyotype analysis, cell culture techniques, immunocytochemistry and real-time PCR.

Key findings: Here we repot the establishment of first CCA cell line, CCA-OF, from a primary tumor of an experimental CCA in Syrian hamsters treated with low doses of dimethyl nitrosamine and associated with *O. felineus* infection. The cell line was found to be allotransplantable. Expression of epithelial and mesenchymal markers (cytokeratin 7, glycosyltransferase exostosin 1, Ca^{2+}-dependent phospholipid-binding protein annexin A1 and vimentin) was demonstrated by immunostaining of the primary tumors, CCA-OF cells, and allotransplants. CCA-OF cells were found to express presumed CCA biomarkers previously detected in both human and experimental tumors associated with the liver fluke infection. The cells were diploid-like (2n = 42–46) with complex chromosomal rearrangements and have morphological features of epithelial-like cells. The usefulness of the CCA-OF cell model for antitumor activity testing was demonstrated by an analysis of effects of resveratrol treatment. It was shown that resveratrol treatment inhibited the proliferation and the migration ability of CCA-OF cells.

Significance: Thus, the allotransplantable CCA-OF cell line can be used in studies on helminth-associated cholangiocarcinogenesis and for the testing of antitumor drugs.

1. Background

Carcinogenic flatworms *Opisthorchis felineus* (Rivolta, 1884), *O. viverrini* (Poirier, 1886), and *Clonorchis sinensis* (Loos, 1907) are causative agents of hepatic helminthiases, widespread on the vast territories of Southeast and Northern Asia [1–3]. According to recent estimates, more than 45 million people worldwide have the liver fluke infections [2,3]. The human infection is due to consumption of raw or undercooked freshwater fish, which is a part of national cuisines in Thailand, Korea, Russia, and some other countries. Adult parasites colonize bile ducts of the liver and the gall bladder [4–6]. The most dramatic complication of liver fluke infections is bile duct cancer, cholangiocarcinoma (CCA) [7].

The International Agency for Research on Cancer has classified liver flukes *O. viverrini* and *C. sinensis* as Group 1 agents, *i.e.*, agents carcinogenic to humans [8] and as major risk factors of CCA in endemic

Abbreviations: ANXA1, annexin A1; CCA, cholangiocarcinoma; CK7, cytokeratin 7; DMN, dimethylnitrosamine; EXT1, exostosin 1; IC_{50}, half-maximal inhibitory concentration; OF, *Opisthorchis felineus*; RSV, resveratrol; VIM, vimentin.

* Corresponding author.
E-mail addresses: mordvin@bionet.nsc.ru (V.A. Mordvinov), lvovamaria@bionet.nsc.ru (M.N. Lvova), rosa@mcb.nsc.ru (S.A. Romanenko), shilov@bionet.nsc.ru (A.G. Shilov).

https://doi.org/10.1016/j.lfs.2021.119494
Received 23 October 2020; Received in revised form 19 March 2021; Accepted 1 April 2021
Available online 20 April 2021

regions [9]. Carcinogenicity of *O. felineus* is poorly studied [1,10]. Nevertheless, the accumulated data on the animal models indicate that this liver fluke has a carcinogenic potential [1,11–15].

CCA is an aggressive cancer characterized by rapid progression and active metastasis. In early stages of the disease, clinical manifestations of CCA are nonspecific, *e.g.*, weight loss, jaundice, and cholangitis. The absence of specific markers of an early stage of this cancer makes it difficult to diagnose CCA [16–21]. Currently, radical surgery is the only treatment that gives hope of recovery to patients with CCA [22]. After the resection, the 5-year survival threshold is overcome by approximately 30–50% of the patients [23]. In addition, CCA often shows chemoradioresistance, which means an urgent need to develop new drugs for the treatment of this tumor [24].

CCA cell lines are successfully used to investigate the biology of cancer and remain the first step for all preclinical studies [16–21]. Considering that the tumors associated with the liver fluke infection and those not associated with it have different transcriptomes and genomic and epigenomic features [25], the need to create cell lines associated with this infection is obvious. Although some CCA cell lines related to *O. viverrini* infection have been established and used in cancer research [26,27], there are still no cell lines derived from tumors associated with *O. felineus* infection. Taking into account the differences in the pathogenesis of helminthiases and in the carcinogenic potential among different species of liver flukes [10,28], it becomes obvious that it is necessary to create a cell model suitable for basic and translational research on the cholangiocarcinogenesis associated with opisthorchiasis felinea.

The aim of this study was to establish and characterize a cell line derived from an experimental CCA associated with *O. felineus* infection. A tumorigenic cell line derived from a hamster CCA will allow researchers to use immunocompetent animals in *in vivo* experiments, which is impossible with cell lines derived from human tumors.

2. Materials and methods

2.1. Hamsters and worms

Two-month-old male golden Syrian hamsters (*Mesocricetus auratus*) from the Specific Pathogen-Free (SPF) Animal Facility of the ICG SB RAS were used for this study.

O. felineus metacercariae were extracted from naturally infected freshwater fish (*Leuciscus idus*) net-caught in the Ob River near Novosibirsk (Western Siberia, Russia) by a research assistant, Viktor Antonov (ICG SB RAS). *O. felineus* metacercariae were extracted as described previously [29,30]. After several washes with normal saline, metacercariae were identified under a light microscope.

All the procedures were in compliance with EU Directive 2010/63/EU for animal experiments. Study design protocols and standard operating procedures (on the hamsters and fish) were approved by the Committee on the Ethics of Animal Experiments of the ICG SB RAS (Permit number 7 of 19 December 2011). The hamsters were euthanized using carbon dioxide.

2.2. Experimental design of CCA formation

Twenty-two male golden Syrian hamsters were randomly subdivided into four groups: (1) untreated animals, (2) dimethylnitrosamine (DMN) administration, (3) *O. felineus* infection, (4) DMN administration + *O. felineus* infection. The animals were infected with 50 metacercariae *via* a gastric tube. The hamsters received a 12.5 ppm aqueous solution of DMN *ad libitum*. The duration of the experiment was 30 weeks. All the hamsters were observed daily for signs of illness, injury, or abnormal behavior by trained personnel of the SPF Animal Facility. Food and water availability and the macroenvironment (temperature, humidity, noise, light intensity, and cleanliness) were also evaluated daily. No unexpected deaths of animals were registered during this study.

2.3. Establishment of an O. felineus–associated hamster CCA cell line

The cell line was obtained from the tissues of the CCA in the Syrian hamsters (Group 4; DMN administration + *O. felineus* infection). Extracted tumor fragments were washed twice in PBS and crushed into small pieces. The resulting suspension was cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12 at 1:1) (Sigma, USA) containing 10% of fetal bovine serum (Thermo Scientific, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in an atmosphere with 5% CO_2 content. During the adaptation period, about 30 passages were performed. At low passages, the cell culture looked morphologically heterogeneous. As the passage number increased, the morphological heterogeneity of the cell culture reduced. The cell line became homogeneous. The cells became epithelial-like polygonal shape with large nuclei. The cells were cultured for more than 70 passages and more than 6 months. The resultant cell line was named CCA-OF.

2.4. Experimental design of tumorigenicity assessment

To study tumorigenic properties of CCA-OF cells, another batch of twenty 2-month-old male hamsters was randomly subdivided into four additional groups. The first group (5 hamsters) was injected with (0.1 mL) sterile saline. The three other groups (5 animals each) were injected with different numbers (1×10^6, 5×10^6, or 10×10^6) of the tumor cells (in 0.1 mL of sterile saline) under thigh skin. One week after the allotransplantation, the volume of tumors in the animals was measured as described previously [31]. The tumors were checked twice a week. Six weeks after the allotransplantation, the hamsters were euthanized. Tumors were excised and analyzed by histological and immunohistochemical methods.

2.5. Histology and immunohistochemistry

For histological examination, the tissue samples were placed in 10% PBS-buffered formaldehyde and processed as described elsewhere [12,32]. The resulting 3.5-μm-thick tissue sections were subjected to a standard staining with hematoxylin and eosin or Van Gieson staining [12,32].

For immunohistochemical analysis, sections were fixed on polylysine glasses, and standard sample preparation was performed: dewaxing in a gradient of ethanol and xylene, permeabilization in 0.01 M citrate buffer (pH 6.0), and processing in 3% H_2O_2 to block endogenous peroxidase activity. Protein antigen blocking and an immunoperoxidase reaction were performed using the Polyvalent HRP Kit (Spring Bioscience, USA). The primary antibodies used in this study were anti-cytokeratin 7 (1:100, cat. # ab9021, Abcam, USA), anti-annexin A1 (1:100, cat. # ab2487, Abcam), anti-exostosin 1 (1:100, cat. # ab126305, Abcam), and anti-vimentin (1:200, cat. # M3202, Spring Bioscience). The negative control was processed in the same way, but the primary-antibody treatment step was skipped. The histological analysis was performed under an AxioImager A1 light microscope (Carl Zeiss, Germany) equipped with an AxioCam MRc camera (Carl Zeiss).

2.6. Real-time PCR

For the analysis of gene expression, liver tissue samples were collected near the center of the large lobe. Total RNA was isolated using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen, USA) and was treated with DNase I (Thermo Scientific). The quality of the RNA was evaluated on a Nanodrop 2000 device (Thermo Scientific) and BioAnalyzer (Applied Biosystems, USA). cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

To study the level of mRNA expression, the following primers were utilized (5′-3′): *Krt7*_F AGCTTCCCCGGATCTTTGAG, *Krt7*_R CGTCC TTCTTCAGCAACACAAAC, *Anxa1*_F AGATGCCAGGGCTTTGTATGA,

*Anxa1*_R TCAGCAAAGAAAGCTGGGGT, *Ext1*_F CGTCCTGCCACAGTATTCG, *Ext1*_R GCTGCTCATAACCTTGCTCTC, *Vim*_F CCAAGTTTGCCGACCTCTCT, and *Vim*_R CATCCACTTCGCAGGTGAGT. The levels of expression of the studied genes were measured relative to *Gapdh* (primers "F" GAACATCATCCCTGCATCCACT and "R" ATGCCTGCTTCACCACCTTCTT).

Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR data were processed in ABI Prism 7000 SDS Software v1.1 (Applied Biosystems). Relative expression levels of the transcripts under study were calculated by the ΔΔCt method.

2.7. Chromosome preparation and staining

Chromosome suspensions were obtained from CCA-OF cell line following previously published protocol with some modifications [33]. Thus, colcemide (KaryoMAX, Gibco) and ethidium bromide were added simultaneously 3 h before harvesting the cells. The hypotonic treatment was carried out for 25 min at 37 °C.

G-differential staining of chromosomes of CCA-OF cells was carried out *via* pretreatment with trypsin [33]. G-differential stained metaphase chromosomes were captured using VideoTest-Karyo and VideoTest-FISH software (Zenit, Saint-Petersburg, Russia) with a JenOptic CCD camera (Jena, Germany) mounted on an Olympus BX53 microscope (Shinjuku, Japan). Images were processed using Paint Shop Photo Pro X3 (Corel, Ottawa, ON, Canada) [34].

2.8. A growth curve and scratch test

The growth curve was obtained by seeding 5×10^3 cells/well in triplicate in a 12-well plate. Viable cells were counted every 24 h using a Goryaev chamber and Trypan Blue staining (Bio-Rad, USA). The experiment lasted for 10 days. The doubling time of the cell population was estimated on a logarithmic scale.

To investigate migration properties, the cells were seeded in a 6-well plate and grown until 90% confluence. An experimental wound was created using a sterile pipette tip. Images were captured after 6, 8, and 10 h by means of an AxioImager A1 inverted microscope (Carl Zeiss) and AxioCam MRc camera (Carl Zeiss). The wound closure and migration rate were calculated in the ImagePro Plus software (Medis Cybernetics, USA).

2.9. The effect of resveratrol (RSV) on CCA-OF cells

Antiproliferative effects of RSV (Sigma) were determined *in vitro*. In brief, 25×10^3 cells were seeded in a 12-well plate overnight. Various concentrations of RSV (6.3, 12.5, 25, 50, 100, or 200 μM) were then added (triplicate wells per concentration) as 0.5% of 200× RSV solutions in DMSO. Control cells were incubated with 0.5% DMSO (Applichem, Germany). All cells were incubated for 72 h. Viable cells were counted using the Goryaev chamber and Trypan Blue staining (Bio-Rad). The IC_{50} value, defined as the concentration of RSV required to decrease the number of cells to 50% at the 72-h time point, was calculated using the 'drc'-package of R (version 3.6.0) [29,30,32].

Antimigration activity of RSV was assessed by a standard scratch test. Cells were seeded in a 6-well plate and grown until 90% confluence. The experimental wound was applied using a sterile pipette tip. Various concentrations (0, 6.3, 25, or 100 μM) of RSV (duplicate samples per concentration) were added to the cells. Images were captured after 6, 8, and 10 h as described above.

2.10. Immunocytochemistry

This analysis was performed using the following primary antibodies: anti-cytokeratin 7 (1:100, ab9021), anti-vimentin (1: 100, cat. # ab11256, Abcam), anti-annexin A1 (1:100, cat. # ab2487, Abcam), and anti-exostosin 1 (1:100, cat. # ab126305, Abcam) and secondary antibodies anti-mouse (1:1000, cat. # SAB4600425, Sigma), anti-rabbit (1:500, cat. # ab150080, Abcam), and anti-goat (1:1000, cat. # ab150129, Abcam) according to the standard protocol. Samples were enclosed in a balm containing DAPI (Sigma), and images were captured by means of the AxioCam MRc camera (Carl Zeiss).

2.11. Statistical analysis

The Mann–Whitney *U* test was performed to compare differences between groups. The statistical analysis was performed in the STATISTICA 6.0 software (Statsoft, USA). Data with a *P* value less than 0.05 were considered statistically significant.

3. Results

3.1. Expression of cytokeratin 7, exostosin 1, annexin A1, and vimentin in the experimental CCA

Experimental CCA tumors were induced in hamsters by the combined action of *O. felineus* and DMN for 30 weeks. The combined effect of *O. felineus* infection and DMN resulted in intrahepatic CCA foci (Fig. 1B) in all animals.

Our immunohistochemical analysis revealed VIM (Fig. 1D) and EXT1 (Fig. 1H) staining of CCA. These markers were not detectable in the liver tissues of untreated animals. By contrast, in the foci of CCA (Group 4 animals, CCA group), both EXT1-positive staining and VIM-positive staining were found (Fig. 1D and H). In addition to the CCA area, an EXT1 signal was present around portal tracts. Intensive VIM staining was documented in the areas of cholangiocyte proliferation and periductal fibrosis. We also showed abundant CK7-positive staining of biliary epithelial cells in the foci of CCA (Fig. 1F). The number of CK7-positive cells was also significantly higher in the areas of bile duct proliferation, cysts, and cholangiofibrosis.

Additionally, we did not find ANXA1-positive signal in liver tissues of untreated hamsters, in Group 4 animals (CCA group; Fig. 1I), by contrast, ANXA1 staining was clearly detectable (Fig. 1J).

To assess *Krt7*, *Vim*, *Anxa1*, and *Ext1* mRNA expression in the liver and tumor tissues of the experimental animals, we carried out real-time PCR. The highest level of expression of all four genes was observed in liver tissues of Group 4 animals and in their tumors (Fig. 2). Levels of *Krt7* mRNA expression were 250- to 300-fold higher in tumor and liver tissues of Group 4 animals than in the liver of untreated hamsters (Mann–Whitney *U* test, $P < 0.05$ and $P < 0.05$, respectively; Fig. 2A). mRNA expression of genes *Vim* and *Anxa1* was also significantly higher in the tumors and liver tissues of Group 4 animals than in untreated animals. In particular, compared to the control, the level of *Anxa1* mRNA by 35- to 60-fold higher (Mann-Whitney U test: $P < 0.05$; Fig. 2B), and the level of *Vim* mRNA in CCA tissue was 10- to 25-fold higher (Mann–Whitney U test: $P < 0.05$; Fig. 2D). Compared to the control liver tissue, the mRNA level of the *Ext1* gene in the tumor tissue was 2.0–2.5-fold higher (Mann–Whitney *U* test, $P < 0.05$; Fig. 2C).

The CCA-OF cell line: morphology, karyotype, cell migration, and expression of potential CCA biomarkers.

CCA-OF cells were derived from the experimental CCA obtained in this study. At the initiation of the culture, epithelial-like cells adhered to the plastic surface and formed colonies surrounded by fibroblasts. Only negligible proliferation of the epithelial-like cells was observed for the first 6–8 weeks; by contrast, the proliferation of fibroblasts was well pronounced. After this period of adaptation to the *in vitro* conditions, CCA-OF cells started to grow rapidly. Within 15 passages, the epithelial-like cells began to form a continuous monolayer, and the number of fibroblasts gradually decreased, to be finally replaced by the tumor cells.

CCA-OF cells formed an adhesive monolayer with high growth density. Phase contrast microscopy revealed that all these cells have morphological features of epithelial-like cells and form sheets of

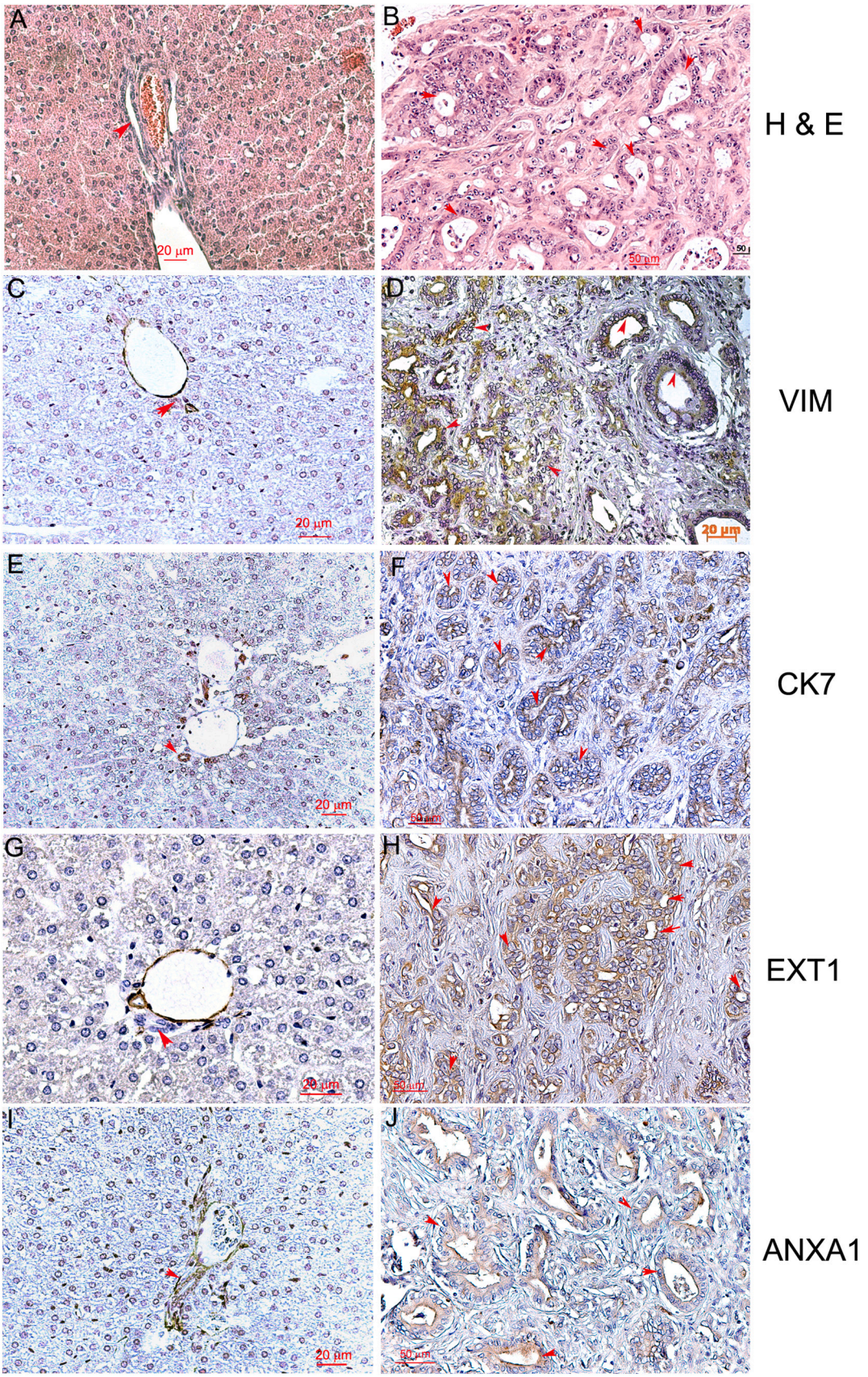

Fig. 1. H&E and immunohistochemical staining of the hamster liver. Biliary histological features observed in the liver from uninfected (Intact: A, C, E, G, and I) and *O. felineus*–infected DMN-treated hamsters (Cholangiocarcinoma). Expression of vimentin (VIM: D), cytokeratin 7 (CK7: F), exostosin 1 (EXT1: H), and annexin A1 (ANXA1: J) is observed in the primary tumor. Bile ducts are pointed out with arrows.

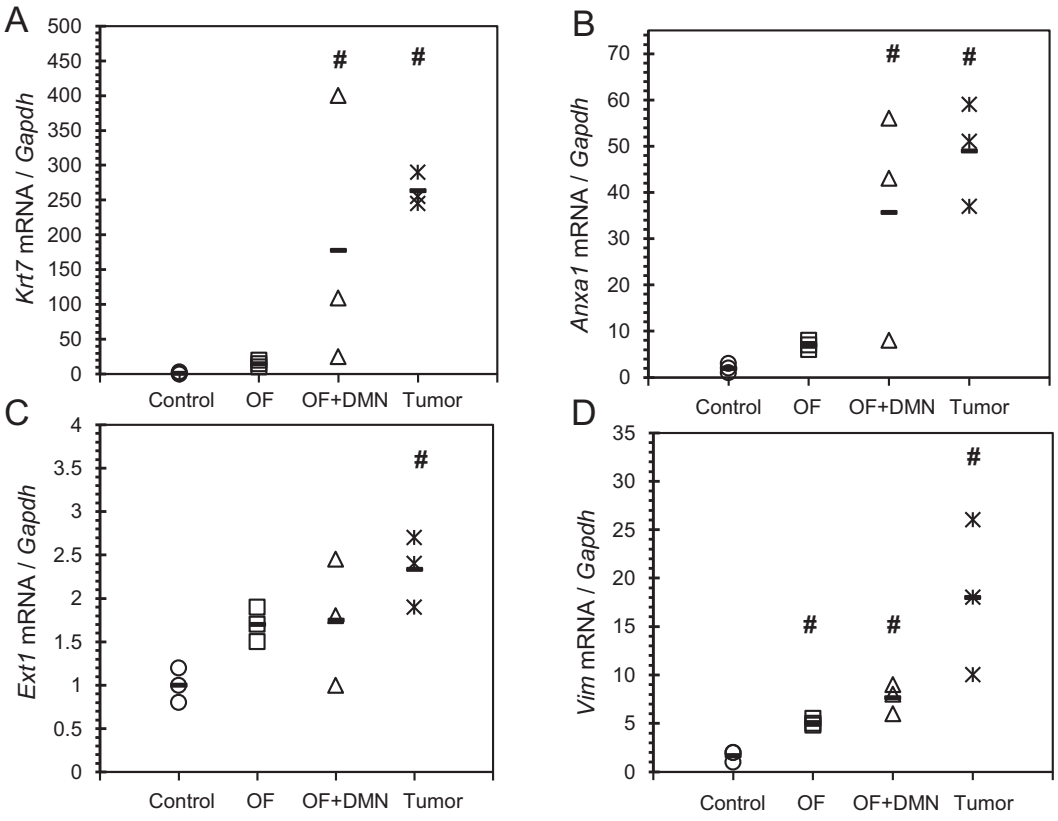

Fig. 2. mRNA expression of presumed CCA marker genes. *Krt7* (A), *Anxa1* (B), *Ext1* (C) and *Vim* (D) mRNA expression in the liver tissue of the laboratory hamsters. Data are presented as a fold-change in gene mRNA expression normalized to the *Gapdh* mRNA level. Lines: average values, dots: individuals. #$P < 0.05$ - as compared to the no-treatment control group.

polygonal cells with large nuclei. We also observed a granularity in the cytoplasm and nuclei of CCA-OF cells (Fig. 3A). Doubling time of the cells, estimated in the exponential phase of growth, was 23.2 ± 2.7 h. CCA-OF cells maintained their morphology under standard cell culture conditions for more than 30 passages during 6 months.

The karyotype analysis revealed that CCA-OF cells were diploid-like; the average number of chromosomes per cell was 44 (range 42–46). Analysis of G-banded chromosomes indicated that the karyotype of CCA-

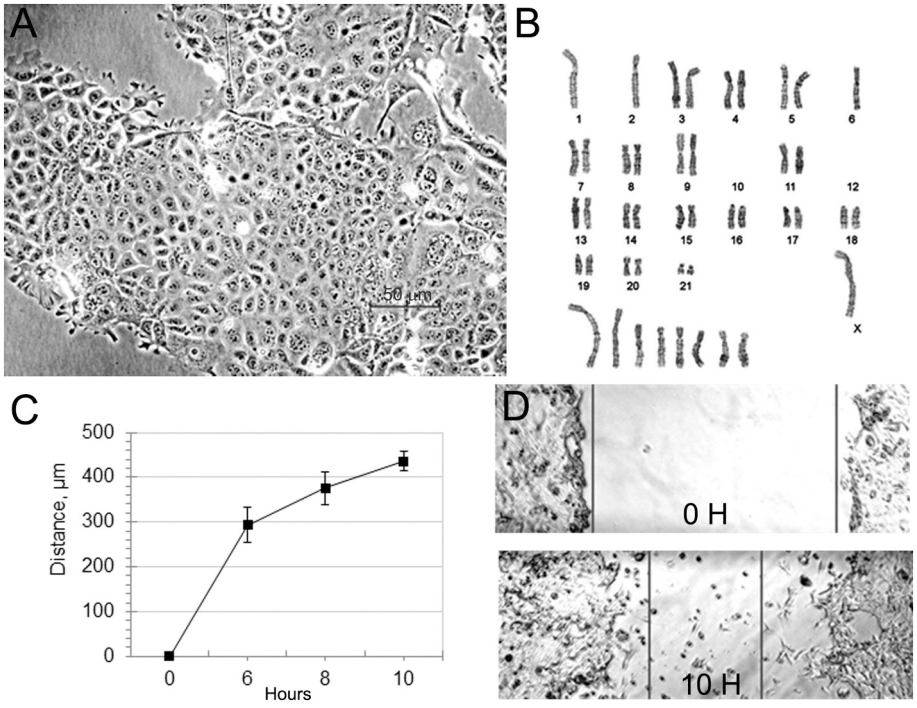

Fig. 3. The CCA-OF cell line. A. Morphology of the cultured cells. An adherent monolayer of CCA-OF cells shows polygonal shapes. B. A representative G-banded karyotype. C, D. The migration ability assessed by the scratch test. Representative pictures are shown. Photographs were taken, and the width of the wound area was measured at the indicated time points. The graphs present the rate of wound healing (%) for each time point, as estimated in ImagePro Plus. Data represent a mean ± SD calculated for three visual fields per condition. *P < 0.05 as compared to the no-treatment control group.

OF cells did not match the standard one for *M. auratus*, and the cell line carried multiple interchromosomal rearrangements (Fig. 3B). A large unpaired submetacentric chromosome was found in the karyotype of this cell line; it was, apparently, a derivative of chromosome 1. Individual homologues in some pairs of chromosomes (for example, pairs 7 and 15) contained additional blocks. Nevertheless, the pattern of G-banding failed to reveal a match of several chromosomes to any chromosomes in the standard karyotype of *M. auratus*.

We evaluated CCA-OF cells' motility in the scratch test. Their type of migration can be described as single-cell migration at a high average motility rate (46.5 ± 1.6 μm/h; Fig. 3C, D).

After stabilization of the growth characteristics of the CCA-OF cell line, we characterized the cells by immunostaining. Positive staining was registered for all tested protein markers, including CK7, VIM, ANXA1, and EXT1 (Fig. 4). This means that CCA-OF cells retained the presumed CCA markers found in the primary tumor.

3.2. The effect of RSV on CCA-OF cells

To assess the usefulness of the CCA-OF cell line for evaluation of anticancer treatments, RSV was selected as a therapeutic agent for this study. As shown in Fig. 5A, RSV inhibited CCA-OF cell proliferation in a dose-dependent manner. RSV significantly inhibited the proliferation of CCA-OF cells starting with 6.3 μM concentration ($P < 0.05$; Mann–Whitney *U* test). Moreover, RSV decreased the number of cells by 91% at its 25 μM concentration ($P < 0.05$; Mann–Whitney *U* test). IC_{50} of RSV was found to be 10.32 ± 0.58 μM.

The results on cell migration distance, as assessed by the scratch test after the treatment with RSV, are presented in Fig. 5B. At 25–100 μM, RSV significantly suppressed the migration ability of CCA-OF cells ($P < 0.05$; Mann–Whitney *U* test).

3.3. CCA-OF allograft transplantation

To check potential tumorigenicity of the CCA-OF cell line, we used an allograft transplantation approach. Different numbers of CCA-OF cells were subcutaneously injected into the hamsters' thigh. Tumor masses were noticeable under thigh skin of all the animals in a week after the allotransplantation. The tumors increased gradually, and tumor volume correlated with the number of injected cells. By the sixth week, the size of the tumors reached 8, 14, and 20 cm^3 in the groups of animals that received 1×10^6, 5×10^6, and 10×10^6 cells, respectively (Fig. 6A). The results of histological analysis suggested that the tumors that grew from the transplanted CCA cells had well-differentiated glandular structure similar to the primary CCA in the hamster liver (Fig. 6B). In addition, immunohistochemical analysis yielded positive immunostaining of the allograft tissues for all the tested (presumed) CCA markers, including ANXA1, EXT1, and CK7 (Fig. 6C–E). These findings indicated that the tumors indeed originated from the transplanted CCA-OF cells and bore a close resemblance to primary CCA in histological and immunohistochemical characteristics of the tumor tissues. Van Gieson staining uncovered abundant elastic fibers in the tumor tissues, thus confirming active formation of the tumor stroma, which is an important indicator of tumor progression (Fig. 6F).

4. Discussion

A novel CCA cell line, CCA-OF—derived from an experimental tumor associated with *O. felineus* liver fluke infection concomitant with DMN administration—has a number of useful features. For instance, the CCA-OF cells proved to be diploid-like, to have morphological features of epithelial-like cells, and to maintain their morphology under standard cell culture conditions during more than 30 passages for at least 6 months. The cell line turned out to be tumorigenic. In particular, after subcutaneous transplantation into hamsters, in all the animals subjected to the allotransplantation, CCA-OF cells were able to form tumors with glandular structure similar to that of the primary CCA in the hamster liver.

According to the Cholangiocarcinoma Experts opinion, no specific serum, urine, biliary, or histological biomarkers are currently available for the diagnosis of CCA [24]. Nevertheless, a combination of known cancer biomarkers may have a diagnostic value for histological

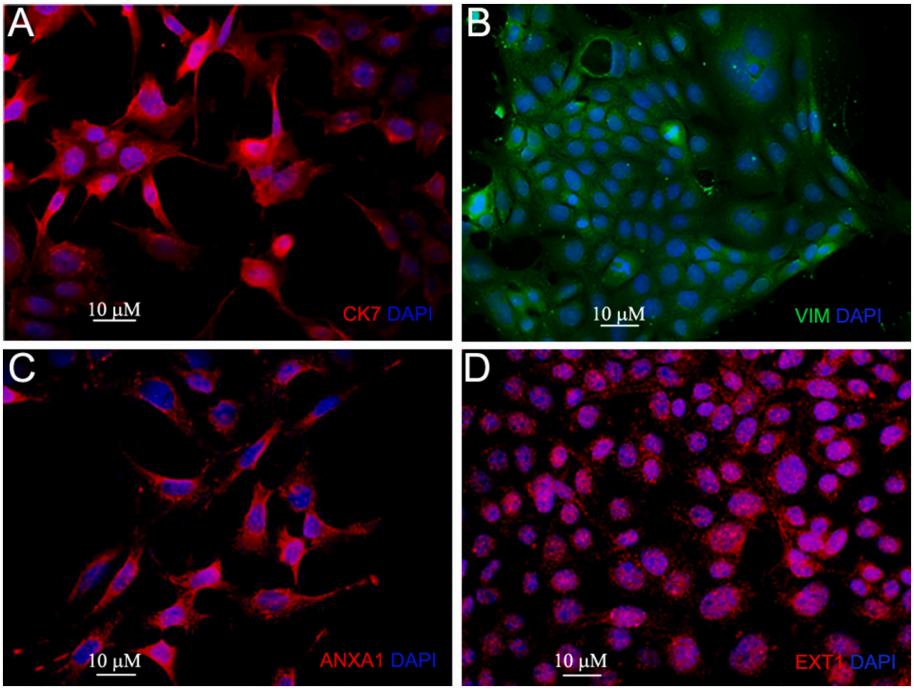

Fig. 4. Immunocytochemical analysis of CCA-OF cells. A. Cytokeratin 7 (CK7). B. Vimentin (VIM), C. Annexin A1 (ANXA1), D. Exostosin 1 (EXT1). The images present staining for specific markers (red and green) and nuclei (labeled with DAPI; blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

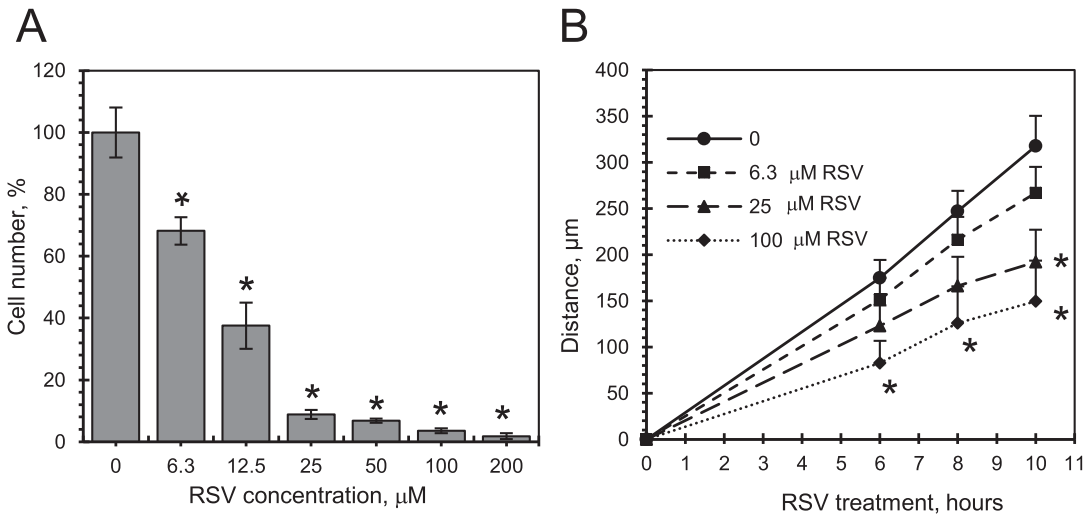

Fig. 5. CCA-OF cells after the treatment with resveratrol (RSV). A. The cell number at 3 days after the treatment with 6.3–200 mM RSV. B. Cell migration distance assessed by the scratch test after the treatment with RSV. Images were captured, and the width of the wound area was measured at the indicated time points. The graphs show the rate of wound healing (%) for each time point, as estimated using the ImagePro Plus software. Data represent the mean ± SD calculated for three visual fields per condition. *P < 0.05 as compared to the no-treatment control group.

identification of CCA [35–39]. Studies on the CCA associated with *O. viverrini* suggest that the expression of genes *Krt7*, *Vim*, *Anxa1*, and *Ext1* is increased in the tumor tissues [19–21,35].

Here, the levels of epithelial (CK7) and mesenchymal (VIM) markers were found to be significantly increased in the tumor tissues of the hamsters with CCA. Moreover, their active expression was retained by CCA-OF cells and by the tumors that resulted from the subcutaneous injection of these cells into hamsters.

EXT1 is an enzyme involved in the synthesis of the heparan sulfate backbone of proteoglycans, which are ubiquitous components of the extracellular matrix. A large amount of EXT1 has been found not only in *O. viverrini*–induced bile duct tumors but also in blood plasma of patients and hamsters with CCA [20]. We detected increased levels of *Ext1* mRNA and of EXT1 in primary CCA, in the CCA-OF cell line, and in the allograft tissues. Expression analysis of another potential CCA marker, ANXA1, yielded similar results. ANXA1 belongs to the annexin family of Ca^{2+}-dependent phospholipid-binding proteins and participates in the regulation of inflammatory-cell activation and some cellular processes [36]. It has been proposed that ANXA1 plays a role in opisthorchiasis-associated cholangiocarcinogenesis [19]. In human studies, this protein is reported to be overexpressed in CCA but underexpressed in hepatocellular carcinoma, suggesting that it may serve as an immunohistochemical marker of CCA [25]. VIM, ANXA1, and EXT1 were detected in primary CCA tissues, CCA-OF cells, and the tumors that grew from the transplanted cells but not in liver tissues of untreated animals.

Studies on the establishment of CCA cell lines associated with the hepatic helminthiases have been conducted for >35 years. The majority of the created cell lines have been derived from the CCA of patients with confirmed opisthorchiasis viverrini. On the other hand, when these cells are transplanted to animals, such animal models of CCA (usually based on immunodeficient mice) have limitations associated with interspecies differences in the tumor microenvironment and a lack of a full-fledged immune response. The hamster model of opisthorchiasis has many similarities with the liver fluke infection in humans, including CCA induction. This observation suggests that the new allotransplantable hamster CCA cell line may be a good model for the investigation of helminth-associated cholangiocarcinogenesis *in vitro* and *in vivo* [40].

The utility of the CCA-OF cell model for antitumor activity testing was demonstrated here by RSV treatment. This compound has been reported to inhibit proliferation and to promote the apoptosis of various cancer cells, including hepatocellular carcinoma cells [41] and human CCA cell lines [27]. Those authors did not provide IC_{50}, and therefore we cannot accurately compare the observed effects; nevertheless, at RSV concentrations of 50–100 μM, the magnitude of the effects on cell motility and proliferation was found to be similar to that in our study.

The CCA-OF cell line shares similarities with a previously described allotransplantable hamster CCA cell line, Ham-1 [40]. This cell line is proposed as a potential model of the CCA associated with opisthorchiasis viverrini. Both CCA-OF and Ham-1 cells are highly tumorigenic, and the tumors that grow out of the transplanted cells retain the morphology and biomarkers of primary CCA. In culture, both cell lines form an adhesive monolayer and show the morphology of epithelial-like cells with a polygonal shape. Increased expression of exostosin 1, annexin A1, and vimentin is often seen in experimental hamster CCA associated with opisthorchiasis viverrini [19,20,42]. This finding suggests that both *O. viverrini* and *O. felineus* can trigger similar mechanisms of cholangiocarcinogenesis. Nevertheless, cell lines CCA-OF and Ham-1 have some marked differences. The doubling time of CCA-OF cells is 1.5-fold shorter than that of Ham-1 cells, 23 *versus* 36 h. In addition, karyotypes of these cells are different: CCA-OF cells are diploid-like, whereas Ham-1 cells are diploid.

Human CCA cell lines derived from the tumors of patients with *O. viverrini*–associated CCA possess distinct characteristics in terms of morphology, modal chromosome number (range), tumorigenicity (tumor formed in xenografted mice), doubling time, and migration and invasion abilities [26]. The hamster CCA-OF cell line resembles some of the known human CCA cell lines induced by the liver fluke *O. viverrini*. For example, KKU-213A, KKU-213B, and KKU-213C cells have tumorigenicity in xenografted mice; the doubling time in culture is close to that of CCA-OF [26]. In terms of migratory properties, CCA-OF cells are close to KKU-213A cells but not to KKU-213C cells. All human and hamster cell lines show detectable expression of epithelial and mesenchymal markers. The hamster CCA-OF cell line has morphological characteristics and phenotype similar to those of human KKU-213A and KKU-213B cell lines.

The main difference between hamster and human cell lines is the ploidy variation. Cell lines derived from hamster CCA are diploid (Ham-1) or diploid-like (CCA-OF). The human cell lines possess aneuploid karyotypes (59–102) with marked structural abnormalities of the chromosomes [26].

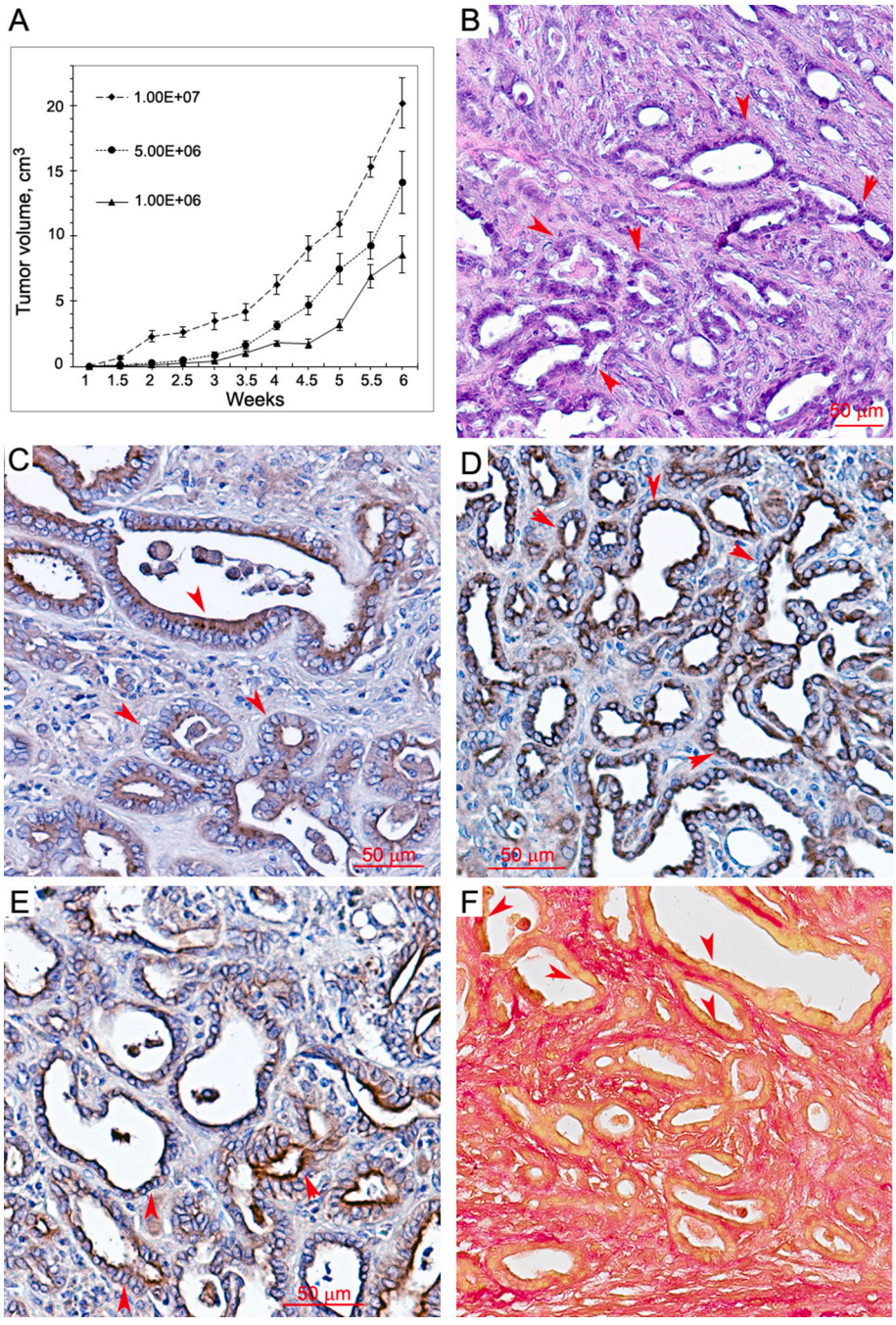

Fig. 6. CCA-OF allograft tumors. A. The tumor size assessed after the injection of CCA-OF cells subcutaneously into the thigh of hamsters in the amount of 1 × 10^6, 5 × 10^6, or 1 × 10^7 cells (n = 5 per group). Tumors formed within a week, and tumor size was measured twice a week. Tumor volume represents mean ± SE. B. H&E staining of an allograft tumor. C–E. Immunohistochemical analysis of an allograft tumor with antibodies against annexin A1 (ANXA1) (C), exostosin 1 (EXT1) (D), and cytokeratin 7 (CK7) (E). F. Van Gieson staining of an allograft tumor. Representative pictures are presented. Bile ducts are indicated by arrows.

5. Conclusion

For future assessment of anticancer treatments, we established the first allotransplantable hamster CCA cell line that is associated with opisthorchiasis felinea. CCA-OF cells express potential CCA biomarkers that have been detected in both human and experimental primary tumors associated with the liver fluke infection. Moreover, the cells are tumorigenic, and the tumors that grew from the transplanted cells retain the morphology and biomarkers seen in primary tumors. In addition, CCA-OF cells have distinct characteristics that distinguish this cell line from a previously described hamster CCA cell line, Ham-1. The advantages of the newly developed CCA cell model were demonstrated here by an analysis of RSV treatment. Altogether, our results suggest that cell line CCA-OF is a suitable model for *in vivo*, *in vitro*, and *ex vivo* studies on general and species-specific mechanisms of cholangiocarcinogenesis associated with *O. felineus* infection, for the search for CCA biomarkers, and for the testing of anticancer drugs.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgements

Microscopy was performed at the Microscopy Center of the ICG SB RAS. We are thankful to Nikolai Shevchuk (http://shevchuk-editing.com) for the English-language editing of this manuscript.

Ethics approval

The experiments were performed in compliance with the EU Directive 2010/63/EU for animal experiments. The animal experiment was approved by the Experimental Animal Ethics Committee at the ICG SB RAS (Permit number 7 of 19 December 2011).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Funding

This work was supported by the Russian Science Foundation [grant number 18-15-00098 to VAM]. The funding agency had no role in this study, *e.g.*, in study design, data collection, or decision to publish.

CRediT authorship contribution statement

MYP and VAM: Conceptualization, Funding acquisition, Project administration, Supervision, and Writing - review & editing; AGS, DVP, OZ, AVK, GAM, SAR, and MNL: Data curation, Formal analysis, and Investigation; AGS, SAR, and AVK: Methodology, Software, and Validation; GAM: Writing - original draft and Visualization. All the authors participated in the drafting of the manuscript and approval of the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.119494.

References

[1] M.Y. Pakharukova, V.A. Mordvinov, The liver fluke *Opisthorchis felineus*: biology, epidemiology and carcinogenic potential, Trans. R. Soc. Trop. Med. Hyg. 110 (1) (2016) 28–36, https://doi.org/10.1093/trstmh/trv085.

[2] T.N. Petney, R.H. Andrews, W. Saijuntha, A. Wenz-Mücke, P. Sithithaworn, The zoonotic, fish-borne liver flukes *Clonorchis sinensis*, *Opisthorchis felineus* and *Opisthorchis viverrini*, Int. J. Parasitol. 43 (12–13) (2013) 1031–1046, https://doi.org/10.1016/j.ijpara.2013.07.007.

[3] R.K. Schuster, Opisthorchiidosis—a review, Infect Disord Drug Targets 10 (5) (2010) 402–415, https://doi.org/10.2174/187152610793180902.

[4] S.A. Be'er, Biology of the Agent of Opisthorchiasis [in Russian], KMK Scientific Press Ltd, Moscow, 2005.

[5] B. Sripa, S. Kaewkes, P.M. Intapan, W. Maleewong, P.J. Brindley, Food-borne trematodiases in Southeast Asia epidemiology, pathology, clinical manifestation and control, Adv Parasitol. 72 (2010) 305–350, https://doi.org/10.1016/S0065-308X(10)72011-X.

[6] E. Mairiang, P. Mairiang, Clinical manifestation of opisthorchiasis and treatment, Acta Trop. 88 (3) (2003) 221–227, https://doi.org/10.1016/j.actatropica.2003.03.001.

[7] W. Saijuntha, P. Sithithaworn, N. Kiatsopit, R.H. Andrews, T.N. Petney, Liver flukes: Clonorchis and Opisthorchis, Adv. Exp. Med. Biol. 1154 (2019) 139–180, https://doi.org/10.1007/978-3-030-18616-6_6.

[8] IARC, IARC working group on the evaluation of carcinogenic risks to humans. Biological agents. A review of human carcinogens, IARC Monogr. Eval. Carcinog. Risks Hum. 100 (B) (2012).

[9] H.R. Shin, J.K. Oh, E. Masuyer, M.P. Curado, V. Bouvard, Y.Y. Fang, S. Wiangnon, B. Sripa, S.T. Hong, Epidemiology of cholangiocarcinoma: an update focusing on risk factors, Cancer Sci. 101 (3) (2010) 579–585, https://doi.org/10.1111/j.1349-7006.2009.01458.x.

[10] N.I. Ershov, V.A. Mordvinov, E.B. Prokhortchouk, M.Y. Pakharukova, K.V. Gunbin, K. Ustyantsev, M.A. Genaev, A.G. Blinov, A. Mazur, E. Boulygina, S. Tsygankova, E. Khrameeva, N. Chekanov, G. Fan, A. Xiao, H. Zhang, X. Xu, H. Yang, V. Solovyev, S.M. Lee, X. Liu, D.A. Afonnikov, K.G. Skryabin, New insights from *Opisthorchis felineus* genome: update on genomics of the epidemiologically important liver flukes, BMC Genomics 20 (1) (2019), 399, https://doi.org/10.1186/s12864-019-5752-8.

[11] M.J. Gouveia, M.Y. Pakharukova, T. Laha, B. Sripa, G.A. Maksimova, G. Rinaldi, P. J. Brindley, V.A. Mordvinov, T. Amaro, L.L. Santos, Costa JMCD, N. Vale, Infection with *Opisthorchis felineus* induces intraepithelial neoplasia of the biliary tract in a rodent model, Carcinogenesis 38 (9) (2017) 929–937, https://doi.org/10.1093/carcin/bgx042.

[12] G.A. Maksimova, M.Y. Pakharukova, E.V. Kashina, N.A. Zhukova, A.V. Kovner, M. N. Lvova, A.V. Katokhin, T.G. Tolstikova, B. Sripa, V.A. Mordvinov, Effect of *Opisthorchis felineus* infection and dimethylnitrosamine administration on the induction of cholangiocarcinoma in Syrian hamsters, Parasitol. Int. 66 (2017) 458–463, https://doi.org/10.1016/j.parint.2015.10.002.

[13] M.Y. Pakharukova, J.M. Correia da Costa, V.A. Mordvinov, The liver fluke *Opisthorchis felineus* as a group III or group I carcinogen, 4open 2 (2019) 23, https://doi.org/10.1051/fopen/2019016.

[14] O.S. Fedorova, Y.V. Kovshirina, A.E. Kovshirina, M.M. Fedotova, I.A. Deev, F. I. Petrovskiy, A.V. Filimonov, A.I. Dmitrieva, L.A. Kudyakov, I.V. Saltykova, P. Odermatt, L.M. Ogorodova, *Opisthorchis felineus* infection and cholangiocarcinoma in the Russian Federation: a review of medical statistics, Parasitol. Int. 66 (4) (2017) 365–371, https://doi.org/10.1016/j.parint.2016.07.010.

[15] M.Y. Pakharukova, O.G. Zaparina, Y.K. Kapushchak, N.V. Baginskaya, V. A. Mordvinov, *Opisthorchis felineus* infection provokes time-dependent accumulation of oxidative hepatobiliary lesions in the injured hamster liver, PLoS One 14 (5) (2019), e0216757, https://doi.org/10.1371/journal.pone.0216757.

[16] W. Loilome, P. Yongvanit, C. Wongkham, N. Tepsiri, B. Sripa, P. Sithithaworn, S. Hanai, M. Miwa, Altered gene expression in *Opisthorchis viverrini*-associated cholangiocarcinoma in hamster model, Mol. Carcinog. 45 (5) (2006) 279–287, https://doi.org/10.1002/mc.20094.

[17] A. Massa, C. Varamo, F. Vita, S. Tavolari, C. Peraldo-Neia, G. Brandi, A. Rizzo, G. Cavalloni, M. Aglietta, Evolution of the experimental models of cholangiocarcinoma, Cancers (Basel) 12 (8) (2020) 2308, https://doi.org/10.3390/cancers12082308.

[18] R. Moll, M. Divo, L. Langbein, The human keratins: biology and pathology, Histochem. Cell Biol. 129 (6) (2008) 705–733, https://doi.org/10.1007/s00418-008-0435-6.

[19] N. Hongsrichan, R. Rucksaken, Y. Chamgramol, P. Pinlaor, A. Techasen, P. Yongvanit, N. Khuntikeo, C. Pairojkul, S. Pinlaor, Annexin A1: a new immunohistological marker of cholangiocarcinoma, World J. Gastroenterol. 19 (16) (2013) 2456–2465, https://doi.org/10.3748/wjg.v19.i16.2456.

[20] J. Khoontawad, N. Hongsrichan, Y. Chamgramol, P. Pinlaor, C. Wongkham, P. Yongvanit, C. Pairojkul, N. Khuntikeo, S. Roytrakul, T. Boonmars, S. Pinlaor, Increase of exostosin 1 in plasma as a potential biomarker for opisthorchiasis-associated cholangiocarcinoma, Tumour Biol. 35 (2) (2014) 1029–1039, https://doi.org/10.1007/s13277-013-1137-9.

[21] P.V. Korita, T. Wakai, Y. Ajioka, M. Inoue, M. Takamura, Y. Shirai, K. Hatakeyama, Aberrant expression of vimentin correlates with dedifferentiation and poor prognosis in patients with intrahepatic cholangiocarcinoma, Anticancer Res. 30 (6) (2010) 2279–2285.

[22] V. Luvira, K. Nilprapha, V. Bhudhisawasdi, A. Pugkhem, N. Chamadol, S. Kamsaard, Cholangiocarcinoma patient outcome in Northeastern Thailand: single-center prospective study, Asian Pac. J. Cancer Prev. 17 (1) (2016) 401–406, https://doi.org/10.7314/apjcp.2016.17.1.401.

[23] T. Patel, Cholangiocarcinoma controversies and challenges, Nat Rev Gastroenterol Hepatol. 8 (4) (2011) 189–200, https://doi.org/10.1038/nrgastro.2011.20.Patel (2011).

[24] J.M. Banales, V. Cardinale, G. Carpino, M. Marzioni, J.B. Andersen, P. Invernizzi, G.E. Lind, T. Folseraas, S.J. Forbes, L. Fouassier, A. Geier, D.F. Calvisi, J. C. Mertens, M. Trauner, A. Benedetti, L. Maroni, J. Vaquero, R.I. Macias, C. Raggi, M.J. Perugorria, E. Gaudio, K.M. Boberg, J.J. Marin, D. Alvaro, Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA), Nat Rev Gastroenterol Hepatol 13 (5) (2016) 261–280, https://doi.org/10.1038/nrgastro.2016.51.

[25] N. Jinawath, Y. Chamgramol, Y. Furukawa, K. Obama, T. Tsunoda, B. Sripa, C. Pairojkul, Y. Nakamura, Comparison of gene expression profiles between *Opisthorchis viverrini* and non-*Opisthorchis viverrini* associated human intrahepatic cholangiocarcinoma, Hepatology 44 (4) (2006) 1025–1038, https://doi.org/10.1002/hep.21330.

[26] B. Sripa, W. Seubwai, K. Vaeteewoottacharn, K. Sawanyawisuth, A. Silsirivanit, W. Kaewkong, K. Muisuk, P. Dana, C. Phoomak, W. Lert-Itthiporn, V. Luvira, C. Pairojkul, B.T. Teh, S. Wongkham, S. Okada, Y. Chamgramol, Functional and genetic characterization of three cell lines derived from a single tumor of an *Opisthorchis viverrini*-associated cholangiocarcinoma patient, Hum. Cell 33 (3) (2020) 695–708, https://doi.org/10.1007/s13577-020-00334-w.

[27] S. Thongchot, A. Ferraresi, C. Vidoni, W. Loilome, P. Yongvanit, N. Namwat, C. Isidoro, Resveratrol interrupts the pro-invasive communication between cancer associated fibroblasts and cholangiocarcinoma cells, Cancer Lett. 430 (2018) 160–171, https://doi.org/10.1016/j.canlet.2018.05.031 (Erratum in: Cancer Lett. 2018;434:206-207).

[28] M.N. Lvova, S. Tangkawattana, S. Balthaisong, A.V. Katokhin, V.A. Mordvinov, B. Sripa, Comparative histopathology of *Opisthorchis felineus* and *Opisthorchis viverrini* in a hamster model: an implication of high pathogenicity of the European liver fluke, Parasitol. Int. 61 (1) (2012) 167–172, https://doi.org/10.1016/j.parint.2011.08.005.

[29] V.A. Mordvinov, A.G. Shilov, M.Y. Pakharukova, Anthelmintic activity of cytochrome P450 inhibitors miconazole and clotrimazole: in-vitro effect on the

liver fluke *Opisthorchis felineus*, Int. J. Antimicrob. Agents 50 (1) (2017) 97–100, https://doi.org/10.1016/j.ijantimicag.2017.01.037.

[30] M.Y. Pakharukova, V.A. Samsonov, E.A. Serbina, V.A. Mordvinov, A study of tribendimidine effects *in vitro* and *in vivo* on the liver fluke *Opisthorchis felineus*, Parasit. Vectors 12 (1) (2019), 23, https://doi.org/10.1186/s13071-019-3288-z.

[31] E.A. Potter, A.S. Proskurina, G.S. Ritter, E.V. Dolgova, V.P. Nikolin, N.A. Popova, O.S. Taranov, Y.R. Efremov, S.I. Bayborodin, A.A. Ostanin, E.R. Chernykh, N. A. Kolchanov, S.S. Bogachev, Efficacy of a new cancer treatment strategy based on eradication of tumor-initiating stem cells in a mouse model of Krebs-2 solid adenocarcinoma, Oncotarget 9 (47) (2018) 28486–28499, https://doi.org/10.18632/oncotarget.25503.

[32] M.Y. Pakharukova, O.G. Zaparina, A.V. Kovner, V.A. Mordvinov, Inhibition of *Opisthorchis felineus* glutathione-dependent prostaglandin synthase by resveratrol correlates with attenuation of cholangiocyte neoplasia in a hamster model of opisthorchiasis, Int. J. Parasitol. 49 (12) (2019) 963–973, https://doi.org/10.1016/j.ijpara.2019.07.002.

[33] S.A. Romanenko, L.S. Biltueva, N.A. Serdyukova, A.I. Kulemzina, V. R. Beklemisheva, O.L. Gladkikh, N.A. Lemskaya, E.A. Interesova, M. A. Korentovich, N.V. Vorobieva, A.S. Graphodatsky, V.A. Trifonov, Segmental paleotetraploidy revealed in sterlet (Acipenser ruthenus) genome by chromosome painting, Mol. Cytogenet. 8 (2015) 90, https://doi.org/10.1186/s13039-015-0194-8.

[34] S.A. Romanenko, E.A. Lyapunova, A.S. Saidov, P.C.M. O'Brien, N.A. Serdyukova, M.A. Ferguson-Smith, A.S. Graphodatsky, I. Bakloushinskaya, Chromosome translocations as a driver of diversification in mole Voles Ellobius (Rodentia, Mammalia), Int. J. Mol. Sci. 20 (18) (2019), https://doi.org/10.3390/ijms20184466 pii:E4466.

[35] S. Saensa-Ard, S. Leuangwattanawanit, L. Senggunprai, N. Namwat, S. Kongpetch, Y. Chamgramol, W. Loilome, W. Khansaard, A. Jusakul, A. Prawan, C. Pairojkul, N. Khantikeo, P. Yongvanit, V. Kukongviriyapan, Establishment of cholangiocarcinoma cell lines from patients in the endemic area of liver fluke infection in Thailand, Tumour Biol. 39 (11) (2017), 1010428317725925, https://doi.org/10.1177/1010428317725925.

[36] Y. Lin, G. Lin, W. Fang, H. Zhu, K. Chu, Increased expression of annexin A1 predicts poor prognosis in human hepatocellular carcinoma and enhances cell malignant phenotype, Med. Oncol. 31 (12) (2014), 327, https://doi.org/10.1007/s12032-014-0327-7.

[37] P. Sharma, S. Alsharif, A. Fallatah, B.M. Chung, Intermediate filaments as effectors of cancer development and metastasis: a focus on keratins, vimentin, and nestin, Cells 23 (5) (2019) 497, https://doi.org/10.3390/cells8050497, 8.

[38] J.P. Thiery, Epithelial-mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (6) (2002) 442–454, https://doi.org/10.1038/nrc822.

[39] M. Kaibori, Y. Kawaguchi, N. Yokoigawa, H. Yanagida, S. Takai, A.H. Kwon, Y. Uemura, Y. Kamiyama, Intrahepatic sarcomatoid cholangiocarcinoma, J. Gastroenterol. 38 (11) (2003) 1097–1101, https://doi.org/10.1007/s00535-003-1203-y.

[40] N. Puthdee, K. Vaeteewoottacharn, W. Seubwai, O. Wonkchalee, W. Kaewkong, A. Juasook, S. Pinlaor, C. Pairojkul, C. Wongkham, S. Okada, T. Boonmars, S. Wongkham, Establishment of an allo-transplantable hamster cholangiocarcinoma cell line and its application for *in vivo* screening of anti-cancer drugs, Korean J Parasitol 51 (6) (2013) 711–717, https://doi.org/10.3347/kjp.2013.51.6.711.

[41] R. Chai, H. Fu, Z. Zheng, T. Liu, S. Ji, G. Li, Resveratrol inhibits proliferation and migration through SIRT1 mediated post-translational modification of PI3K/AKT signaling in hepatocellular carcinoma cells, Mol. Med. Rep. 16 (6) (2017) 8037–8044, https://doi.org/10.3892/mmr.2017.7612.

[42] J. Khoontawad, C. Pairojkul, R. Rucksaken, P. Pinlaor, C. Wongkham, P. Yongvanit, A. Pugkhem, A. Jones, J. Plieskatt, J. Potriquet, J. Bethony, S. Pinlaor, J. Mulvenna, Differential protein expression marks the transition from infection with *Opisthorchis viverrini* to cholangiocarcinoma, Mol. Cell. Proteomics 16 (5) (2017) 911–923, https://doi.org/10.1074/mcp.M116.064576.