Human cytomegalovirus infection enhances 5-lipoxygenase and cycloxygenase-2 expression in colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the most common and fatal types of cancer. Inflammation promotes CRC development, however, the underlying etiological factors are unknown. Human cytomegalovirus (HCMV), a virus that induces inflammation and other cancer hallmarks, has been detected in several types of malignancy, including CRC. The present study investigated whether HCMV infection was associated with expression of the pro-inflammatory enzymes 5-lipoxygenase (5-LO) and cyclooxygenase-2 (COX-2) and other molecular, genetic and clinicopathological CRC features. The present study assessed 146 individual paraffin-embedded CRC tissue microarray (TMA) cores already characterized for TP53 and KRAS mutations, microsatellite instability (MSI) status, Ki-67 index and EGFR by immunohistochemistry (IHC). The cores were further analyzed by IHC for the expression of two HCMV proteins (Immediate Early, IE and pp65) and the inflammatory markers 5-LO and COX-2. The CRC cell lines Caco-2 and LS-174T were infected with HCMV strain VR1814, treated with antiviral drug ganciclovir (GCV) and/or anti-inflammatory drug celecoxib (CCX) and analyzed by reverse transcription-quantitative PCR and immunofluorescence for 5-LO, COX-2, IE and pp65 transcripts and proteins. HCMV IE and pp65 proteins were detected in ~90% of the CRC cases tested; this was correlated with COX-2, 5-LO and KI-67 expression, but not with EGFR immunostaining, TP53 and KRAS mutations or MSI status. *In vitro*, HCMV infection upregulated 5-LO and COX-2 transcript and proteins in both Caco-2 and LS-174T cells and enhanced cell proliferation as determined by MTT assay. Treatment with GCV and CCX significantly decreased the transcript levels of COX-2, 5-LO, HCMV IE and pp65 in infected cells. HCMV was widely expressed in CRC and may promote inflammation and serve as a potential new target for CRC therapy.

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Abbreviations: HCMV, human cytomegalovirus; CRC, colorectal cancer; 5-LO, 5-lipoxygenase; COX-2, cyclooxygenase-2; GCV, ganciclovir; CCX, celecoxib; MSS, microsatellite stability; MSI, microsatellite instability; dpi, days post-infection; IE, immediate early

Key words: colorectal cancer, human cytomegalovirus, inflammation, ganciclovir, celecoxib

Introduction

Colorectal cancer (CRC), the third most common and second deadliest cancer worldwide with an incidence rate of 25.9

and a mortality rate 11.8/100,000 individuals in 2020 (1), is a progressive, multistep disease that develops following phenotypical progression from adenoma to invasive adenocarcinoma. Although environmental and genetic risk factors have been identified (2), the etiology of CRC is unknown. Among environmental factors, inflammatory bowel disease is associated with higher CRC risk (3) and higher inflammatory activity is associated with worse prognosis (4). Of clinical relevance, higher levels of circulating inflammatory markers C-reactive protein, IL-6), and chitinase-3-like-1 protein have been found in patients with CRC compared with controls and increase risk of complications after tumor resection in older patients with CRC (5). The role of inflammation is indicated by evidence that long-term use of aspirin or other non-steroidal anti-inflammatory drugs decreases CRC risk (6-9).

A causal link between inflammation and cancer was suggested in 1863 by Rudolf Virchow, who noted that cancer originates at sites of inflammation (10). Inflammation is considered one of the major drivers of tumor transformation and progression in CRC (11-13) . Cellular inflammation is initiated by activation of phospholipase A2, which releases arachidonic acid (AA) from membrane phospholipids. AA is a polyunsaturated 20-carbon fatty acid metabolized by either the cyclooxygenase (COX) or the lipoxygenase (LO) pathways to generate powerful inflammatory mediators called eicosanoids, which comprise prostaglandins, thromboxanes and leukotrienes. Eicosanoids serve as autocrine/paracrine regulators of inflammation and modulate cancer-associated physiopathological responses, including suppression of immune surveillance and enhanced proliferation and invasiveness of tumor cells (14).

A series of studies indicate that abnormal AA metabolism is involved in cardiovascular and metabolic disease (15) as well as in carcinogenesis (16). Overexpression of COX-2 and 5-LO has been reported in several human malignancies, including CRC (17). High COX-2 and 5-LO expression is associated with more aggressive tumor phenotype and poor survival (17,18). COX-2 can be induced by inflammatory cytokines such as IL-1β, TNF-α and IL-6, which are abundantly expressed in CRC (19). Expression of 5-LO is tightly regulated by promoter methylation (20) and is induced by the demethylating compound 5-aza-2'-deoxycytidine in cancer cell lines (21) or stimulation with lipopolysaccharide, TGFβ and/or 1,25$(OH)_2$D3 in monocytes and differentiating macrophages (20,22).

Several anti-inflammatory agents have anti-cancer properties (23). Long-term treatment with COX-2 inhibitors significantly decreases risk of many malignancies, including breast (24), lung (25) and prostate cancer (26,27) and CRC (28). Similarly, prolonged use of non-specific COX inhibitor aspirin lowers the risk of colorectal adenoma (29) and CRC (30-33), being especially effective in cases with PIK3CA mutations (34) that are found in 10-15% of all CRC cases. Inhibition of 5-LO triggers apoptosis in breast, lung, prostate and colon cancer cell lines (35) and combined use of COX-2 and 5-LO inhibitors have additive anti-CRC effects (36).

Emerging evidence indicates an important role of human cytomegalovirus (HCMV) in cancers of different origins, including CRC (37). HCMV, a β-herpes virus with a 83% worldwide prevalence in 2019 (38), exhibits a complex genome encoding >750 RNAs and 200 proteins, most of which promote viral persistence and replication via dysregulation of cellular and immunological functions (39). Only ~50 viral proteins are considered to be essential for viral replication (40). Studies reported a high prevalence of HCMV antigens and/or DNA in various types of cancer, including CRC (18,41,42), breast (43) and prostate (44) cancer, medulloblastoma (45), neuroblastoma (46) and glioblastoma (47). HCMV DNA and/or protein is generally not detectable or is expressed at low levels in adjacent non-neoplastic tissue, which raises the question of the relevance of this virus in tumor biology.

HCMV infection is generally asymptomatic but may cause severe disease in immunocompromised patients. Following primary infection, the virus establishes latency in myeloid cells, where it can be reactivated by inflammation (48,49). This may be autonomously promoted via virus-encoded chemokine receptor homologue US28, an inducer of the NF-κB/COX-2/VEGF axis (50,51). Our previous study showed that HCMV infection induces expression of 5-LO and production of leukotriene B4 (LTB4, 52). Our previous study reported that HCMV induces COX-2 and 5-LO expression in breast cancer cells (53) and HCMV infection is associated with COX-2 and 5-LO expression in breast tumors and poor patient outcome (54); there is also a correlatin between presence of HCMV proteins and 5-LO expression in borderline ovarian tumor (55). Furthermore, COX-2 inhibitor celecoxib (CCX), in combination with the antiviral drug valganciclovir (VGCV), decreases growth of HCMV-positive medulloblastoma in a xenograft model and inhibits prostaglandin E2 (PGE2) production (45).

In 2002, Harkins *et al* (18) analyzed colorectal adenoma, CRC and adjacent non-neoplastic mucosa samples from 29 patients and found that the early and late HCMV proteins immediate early 1-72 (IE1-72) and pp65 were present in 82 and 78% of the adenomas and 80 and 92% of the adenocarcinomas, respectively. The presence of HCMV in cancer cells was confirmed by detection of HCMV DNA by *in situ* hybridization (ISH) and PCR. Follow up studies have reported that HCMV proteins are frequently present in CRC and HCMV DNA is found in tumors but rarely in non-tumorous mucosa (41,56). Meta-analyses suggest an increased risk of CRC in patients with HCMV (56,57). In older patients, HCMV is linked to worse outcome, regardless of stage (58) and high levels of HCMV-specific immunostaining in primary tumors are correlated with shorter survival and risk of brain metastasis (54). The relationship between CRC and inflammation is consistent with a potential effect of HCMV in colorectal tumorigenesis. HCMV US28 induces intestinal tumors in transgenic mice (59) and HCMV infection induces BCL-2 and COX-2 protein expression in Caco-2 cells (18).

The present study aimed to analyze the expression of IE and late HCMV protein pp65, together with 5-LO and COX-2 by immunohistochemstry, in a pathologically and genetically characterized tissue microarray (TMA) series of 146 CRC cases. It was hypothesized that HCMV is a major driver of inflammation in CRC and a possible target of therapy.

Materials and methods

CRC cases. The study was part of 'Epidemiology of colorectal cancer and its risk factors in Iran', approved by the

Institutional Review Board of the Digestive Disease Research Center, Tehran University of Medical Sciences, Tehran, Iran (approval no. FWA00001331/DHHS-IRB00001641; March 17, 2004). The series of 146 individual, formalin-fixed, paraffin-embedded CRC cores were derived from patients who underwent surgical resection from February 1998 to September 2003 in two major hospitals (Atieh Hospital and Mehr General Hospital), in Tehran, Iran (median age at surgery 53 years; range 20-80 years). Patients included 50 (42.4%) females and 60 (57.6%) males. The cases were previously characterized for Duke's staging, differentiation grade (poor/moderate/well-differentiated), microsatellite instability (MSI)/microsatellite stable (MSS) status, tumor-associated KRAS and TP53 gene mutations (60,61) and Ki-67 and EGFR immunostaining (62). TMA blocks (2 mm cores representative of morphologically relevant areas containing ≥50% tumor cells) were obtained at the Pathology Section of the Center for Advanced Studies and Technology, G. d'Annunzio University, Chieti, Italy, as previously described (63) and CRC diagnosis was confirmed after pathological review. Patients had no history of inflammatory bowel disease, familial adenomatous polyposis or hereditary nonpolyposis CRC and had not undergone preoperative chemotherapy or radiotherapy. Loss of cores due to progressive exhaustion after sectioning account for variations of the total number of cases stained for each marker.

Immunohistochemical staining. TMA blocks were serially cut at a diameter of 5 μm and immunostained as previously reported (54,55,64). Sections were deparaffinized in xylene (Sigma Aldrich; Merck KGaA), rehydrated in descending ethanol gradient and washed in TBST (0.1% Tween 20, Sigma-Aldrich; Merck KGaA). Antigen retrieval was performed by heating in Citra plus pH 6.0 buffer in a pressure cooker (Biocare Medical, LLC) at 95˚C for 20 min. Endogenous peroxidase was blocked with 3% H_2O_2 (Histolab Products AB) for 15 min at room temperature in the dark. Endogenous avidin and biotin were neutralized using Avidin-Biotin Blocking kit (Dako; Agilent Technologies, Inc.). Slides were incubated in Protein Block (Dako; Agilent Technologies, Inc.) for 30 min at room temperature, then overnight at 4˚C with primary antibodies against HCMV IE (1:300; Cat. No. MAB819R, Merck KGaA), pp65 (1:100; Cat. No. NCL-CMVpp65, Leica Biosystem), COX-2 (1:400; Cat. No. 12282, Cell Signaling Technology, Inc.), 5-LO (1:200, Cat. No. ab169755 Abcam) diluted in antibody diluting buffer (BioGenex Laboratories). The slides were washed in TBST, incubated with secondary anti-mouse (1:20; Cat. No. HK325-UM, BioGenex Laboratories) or anti-rabbit (1:20; Cat. No. HK326-UR, BioGenex Laboratories) antibody for 30 min at room temperature, then washed again in TBST and incubated with streptavidin-biotin-peroxidase complex for up to 5 min at room temperature (1:20; cat. No. HK320-UK,BioGenex Laboratories). Hematoxylin (ready to use, Histolab) was used for counterstaining for up to 10 seconds at room temperature. HCMV-infected human lung and HCMV-negative human tonsil (positive and negative controls, respectively). To assess cancer specificity, HCMV IE, pp65, COX-2 and 5-LO immunostaining was performed on five individual non-tumorous colorectal mucosa samplesrun in parallel to the CRC cases. The non-tumorous colorectal mucosa samples were previously collected at the Department of Molecular Medicine and Surgery at Karolinska Institutet and ethical approval was granted by Regional Human Ethics Committee, Stockholm, Sweden (approval no2008/628-31). Isotype controls for primary antibodies targeting HCMV-IE, HCMV pp65, COX-2 and 5-LO were also run in all experiments. Slides stained for Ki-67 and EGFR as previously described (62) were re-analyzed, with the newly stained tissues. Serial sections were digitally scanned using a Hamamatsu Nano Zoomer-XR Digital slide scanner (Hamamatsu Photonics K.K.) and shared for analysis at CAST and Bioclinicum Karolinska Institutet using Nano Zoomer Digital Pathology (NDP) viewer software (Cat. No. U12388-01; Version NDP. view2 Viewing, Hamamatsu). Digital sections were evaluated independently by a pathologist and senior researcher to assess percentages of cells expressing HCMV IE, pp65, COX-2, 5-LO, KI-67 and EGFR proteins. The scoring system was as follows: Negative (0%), 1 (<25%), 2 (25-50%), 3 (51-75%) and 4 (>75%). Some tissue cores were lost during the staining procedure.

Cell culture. Caco-2 (cat. no. HTB-37) and LS-174T (cat. no. CL-188) cells, selected to represent a differentiation-competent and poorly differentiated, highly invasive human CRC cell line (65), were acquired from American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 100 U/ml penicillin and streptomycin at 37˚C in 5% CO_2/95% air. Cells were infected at 50% confluency with HCMV strain VR1814 at multiplicity of infection (MOI) 5 and were collected for analysis at 1, 3 and 7 days post-infection (dpi). Mock-infected cells were used as controls.

In vitro treatment. A total of ~1.5x10^5 Caco-2 and LS-174T cells were plated in 6-well plates in the presence or absence of 100 μM ganciclovir (GCV; Roche Diagnostics) and/or 10 μM CCX (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C, then infected with HCMV strain VR1814 at MOI 5 or mock infected (cell culture medium not containing viral particles). GCV was used instead of its pro-drug form VGCV, as VGCV needs to be hydrolyzed into GCV to exert its antiviral activity. This is mediated by cells in in the liver . *In vivo*, GCV has to be administered intravenously/intraperitoneally while VGCV is taken perorally. GCV is already active and the only suitable drug form for *in vitro* experiments and has been used in multiple *in vitro* studies (66,67). At 1, 3 and 7 dpi, the cells were lysed and collected with TRIzol (Thermo Fisher Scientific, Inc.). Samples were stored at -80˚C for up to 1 month.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). RNA of treated cells was extracted using Trizol manufacturer's protocol (Thermo Fisher Scientific, Inc.). RNA concentration was measured using Nanodrop (Thermo Fisher Scientific, Inc.).

A total of ~1 μg RNA was used for cDNA synthesis using SuperScript™ III First-Strand Synthesis System according to manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.) and reactions were primed with random

hexamers. Expression of target genes was assessed by TaqMan PCR using primers from Thermo Fisher Scientific, Inc. as follows: IE forward, 5'-GTGACCCATGTGCTTATGACT CTAT-3' and reverse, 5'-CTCAACATAGTCTGCAGGAAC GT-3', probe FAM-TTGGTCACGGGTGTCTC), HCMV tegument protein pp65 (on-demand, forward primer 5'-CCCAGC GTGACGTGCATAA-3' and reverse primer 5'-AGGTGTACC TGGAGTCCTTCTG-3', probe FAM-CTCCGGCAAGCT CT), COX-2 (sequence not available; Cat. No. hs00153133.m1) and 5-LO (sequence not available; Cat. No. hs00167536.m1). PCR mixes were prepared using the Applied Biosystems™ TaqMan™ Fast Universal PCR Master Mix (cat. No. 10702697, Applied Biosystems, Thermo Fisher Scientific, Inc.). The 7900HT Fast Real-Time PCR System (Cat. No. 4329001, Applied Biosystems, Thermo Fisher Scientific, Inc.) was used to process the PCR plates with the thermocyling conditions set according to the manufacturer instruction (denaturation at 95˚C for 20 seconds, annealing 95˚C for 1 second, extension 60˚C for 20 seconds, 40 cycles). The results were analyzed with SDS version 2.4 software (Cat. No. 4350490, Applied Biosystem). The $2\text{-}\Delta\Delta Cq$ method (68) was used to quantify the relative expression of the targets normalized to the housekeeping gene human β2-microglobulin (sequence not available; cat. No. hs00187842.m1).

Immunofluorescence (IF). HCMV-infected CRC cells LS-174T and Caco-2 were seeded at a density of 10^4 cells/well and cultured overnight on sterile eight-well chamber glass slides to allow cells to attach, fixed with ice-cold methanol:acetone (1:1) for 20 min at -20˚C, rinsed with PBS and incubated with Dako protein Blocker (ready to use; Agilent) and Fc receptor blocker (Innovex Biosciences Inc.; both 30 min at room temperature). After blocking, cells were incubated overnight at 4˚C with rabbit anti-human 5-LO (1:500; Cat. No. ab169755, Abcam), anti-COX-2 (1:500; Cat. No. 12282, Cell Signaling Technology, Inc.) and mouse anti-HCMV IE (1:1,000; Cat. No. 11-003; Biomerieux). The primary antibodies were detected by incubating cells with secondary antibodies (Alexa Fluor 594 donkey anti-mouse; Cat. No. R37115 and Alexa Fluor 488 donkey anti-rabbit Cat. No. A-21206; both 1:500; both Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h and DAPI for 5 min at room temperature (Sigma-Aldrich; Merck KGaA). After mounting slides with fluorescence mounting medium (Dako Cytomation), confocal microscopy at 20x was performed using Zeiss LSM 700 confocal microscope. Rabbit IgG1 isotype control (1:300; Cat. No. AB-105-C, R&D Systems, Inc.) and mouse monoclonal IgG2a (1:300, Cat. No. ab190463 Abcam) served as negative controls.

MTT assay. A total of ~$3\text{x}10^3$ LS-174T and Caco-2 cells were seeded onto 96-well plates and assayed for proliferation at 1, 3 and 7 dpi, either with HCMV VR1814 at MOI 5 or mock, using MTT kit (Cat. No. 11465007001; Sigma Aldrich) according to the manufacturer's protocol. Absorbance was read at 570 nm with a reference wavelength of 670 nm with a Versa Max Plate reader (Molecular Devices, LLC).

Viral infectivity assay. LS-174T, Caco-2 and MRC5 cells (ATCC; cat. no. CCL-171) were seeded in a six-well plate at 10^5 cells/well and incubated at 37˚C. The MRC-5 cells were used as a positive control for the viral infectivity assay since they are used in routine virus propagation protocols, microbiological diagnostic, and sustain a lytic infection of HCMV in which particles are produced (69,70). RPMI 1640 (Thermo Fisher Scientific, Inc.) was used to culture LS-174T, Caco-2 and EMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 100 U/ml penicillin and streptomycin was used for MRC-5 cells.

At 24 h after seeding, cells were infected either with HCMV-VR1814 at MOI 5 for colon cancer cell lines and MOI 1 for MRC5 or with mock controls. At 7 dpi, supernatants were harvested from HCMV-infected and mock-infected LS-174T, Caco-2 and MRC5 cells and underwent centrifugation at 1,200 g for 10 min at room temperature. A total of 2 ml supernatant from one well of infected MRC5 or colon cancer cells was used to directly infect for 6 h at 37˚C in a cell culture incubator a chamber slide of uninfected MRC5 cells seeded 24 h prior at a density of $2\text{x}10^3$ cells/well. Supernatant from infected MRC5 cells was incubated in a humidified chamber at 37˚C with 5% CO_2 for 6 h. Cells were washed three times with sterile PBS to remove the supernatants. Fresh RPMI for LS-174T, Caco-2 and EMEM for MRC-5 was added to each well to a total volume of 0.5 ml/well. At 7 dpi, supernatants were washed with PBS, fixed in Paraformaldehyde (PFA) 4% in PBS for 20 min at room temperaturend then washed again in PBS. Cell membranes were permeabilised in 0.2% Triton X-100 (Sigma-Aldrich) for 5 min. The cells were washed with PBS and incubated with Dako protein Blocker (ready to use; Agilent) and Fc receptor blocker (Innovex Biosciences; both 30 min at room temperature). After blocking, cells were incubated overnight at 4˚C with mouse anti-HCMV IE (1:1,000; Cat. No. 11-003, Argene, Biomerieux). The primary antibodies was detected by incubating cells with secondary antibody (Alexa Fluor 594 donkey anti-mouse Cat. No. R37115 1:500; Invitrogen, Thermo Fisher Scientific, Inc.) at room temperature for 1 h and DAPI for 5 min at room temperature (Sigma-Aldrich; Merck KGaA). After mounting slides with fluorescence mounting medium (Dako Cytomation), confocal microscopy at 20x was performed using Zeiss LSM 700 confocal microscope. Mouse monoclonal IgG2a (1:300, Cat. No. ab190463 Abcam) served as negative controls.

Statistical analysis. Correlation between categorical ordinal (IHC score) and continuous variables (age) was calculated by multivariable non-parametric Spearman analysis. Associations between binominal values (sex, MSI, TP53 and KRAS mutation) and HCMV, 5-LO and COX-2 IHC score were calculated by Fisher's exact test. IHC scores were grouped into 'low positivity' group (scores 0-2, <50% tumor cells stained positive) and 'high positivity' group (scores 3 and 4; >50% tumor cell stained positive). One-way ANOVA test followed by Tukey's post hoc test was used to analyze RT-qPCRdata. Data are presented as the mean ± standard deviation. At least 3 indipendent experimental repeats were run. All statistical hypotheses were two-sided. $P \leq 0.05$ was considered to indicate a statistically significant difference. GraphPad Prism (version 9.2, GraphPad Software, Inc.; Dotmatics) was used for statistical analysis.

Table I. Immunohistochemical staining score in colorectal cancer cases.

Target protein	Negative (%)	1 (%)	2 (%)	3 (%)	4 (%)	Total (%)
HCMV IE	7 (4.9)	12 (8.3)	25 (17.4)	51 (35.4)	49 (34.0)	144 (100)
HCMV pp65	20 (14.1)	40 (28.2)	24 (16.9)	38 (26.8)	20 (14.1)	142 (100)
5-LO	2 (1.4)	5 (3.5)	15 (10.3)	33 (22.8)	90 (62.1)	145 (100)
COX-2	7 (3.5)	32 (22.1)	29 (20.0)	39 (26.9)	37 (25.5)	145 (100)
Ki-67[a]	13 (9.3)	13 (9.3)	38 (27.1)	42 (30.0)	34 (24.3)	140 (100)
EGFR[a]	80 (55.2)	45 (31.0)	16 (11.0)	4 (2.8)	0 (0.0)	145 (100)

The scoring system was as follows: Negative, 0%; 1, <25%; 2, 25-50%; 3, 51-75% and 4, >75%. HCMV, human cytomegalovirus; IE, immediate early; pp65, posphoprotein 65; LO, 5-lipoxygenase; COX, cyclooxygenase-2. [a]Ki-67 and EGFR staining had already been performed in a previous study (62).

Results

Demographic, clinical and histopathological characterization of CRC cases. TMA series of 146 Iranian CRC cases was analyzed. Demographic and histopathological characteristics are summarized in Table SI. The cases were previously characterized for Duke's staging, differentiation (poor/moderate/well-differentiated), MSI status and KRAS and TP53 mutation (60,61). A total of two tumors (0.2%) were rated as Duke's stage A, 65 (55.1%) as stage B, 43 (36.4%) as stage C and 8 (6.8%) as stage D. Most tumors showed moderate differentiation (84, 71.2%) while 17 were well differentiated (14.4%) and 17 were poorly differentiated (14.4%). Mutations in KRAS and TP53 were detected in 41/113 (36.3%) and 52/117 (44.4%) cases, respectively; 28 cases were MSI$^+$ (23.7%) and 90 cases were MSS (76.3%).

Immunohistochemical staining for nuclear Ki-67 and cell membrane EGFR were performed previously (62); IHC data are summarized in Table I. Canonic nuclear Ki-67 was expressed in 127 of 140 (90.7%) cases, of which 76 (54.3%) had >50% Ki-67-positive cancer cells (IHC score 3 and 4). EGFR exhibited canonical membranous localization in 65/140 (46.4%) of cases. Representative images for EGFR and Ki-67 immunostaining are shown in Fig. S1.

High expression of HCMV and inflamatory proteins is found in CRC. A total of 146 individual TMA cores were available for HCMV IE, HCMV pp65, COX-2 and 5-LO immunostaining. Some cores were lost during staining. As sumarized in Table I, HCMV IE expression was detected in 137 of 144 (95.1%) individual cores, of which 51 (38.3%) scored 3 and 49 (30.8%) 4. HCMV pp65 was detected in 122 of 142 (85.9%) biopsies, approximately half of which scored 1 or 2 (n=40 and n=24, respectively) and the rest had score of 3 (n=38) or 4 (n=20). IE and pp65 proteins were predominantly found in the cytoplasm of tumor cells (Fig. 1A, B, D and E) and were not detectable in non-tumorous mucosa (Fig. 1C and F respectively). In certain cases, IE was also found in endothelial cells (Fig. 1A) and pp65 in tumor-associated interstitial cells (Fig. 1D). COX-2 was localized in the cytoplasm and expressed in 137 of 144 (95%) individual cores: 32 (22.2%) had score 1, 29 (20.1%) score 2, 39 (27%) score 3 and 37 (25.7%) score 4 (Fig. 2A, B). No COX-2 immunostaining was found in non-tumorous mucosa (Fig. 2C). Canonical nuclear 5-LO staining was observed in 143 of 145 (96.6%) individual cores (Fig. 2D and E), most of which exhibited a high grade (90, 62.9% scored 4), while no immunostaining was apparent in non-tumorous mucosa (Fig. 2F).

Expression of HCMV proteins is correlated with inflammatory and proliferation markers. Spearman correlation tests were performed for immunohistopathological and clinical variables (Table II). The expression of IE and pp65 was correlated positively in the tumor tissue specimens. The inflammatory marker COX-2 was positively correlated with both IE and pp65. Positive correlations were also observed between 5-LO and IE and and pp65. Additionally, expression of viral proteins IE and pp65 was correlated with the proliferation marker Ki-67, suggesting that increased cellular proliferation might be linked to higher viral load. No correlation was found between IE or pp65 and Duke's stage, histological grade, EGFR expression and age. 5-LO and COX-2 staining was correlated positively (P<0.0001). COX-2 staining was positively correlated with Ki-67 labeling; 5-LO was not significantly positively correlated with Ki-67. A higher differentiation grade was correlated with older age.

To investigate whether HCMV and inflammatory protein expression was correlated with sex, MSI status and mutations in TP53 or KRAS, CRC samples stained for HCMV IE, HCMV pp65, 5-LO and COX-2 by IHC were grouped according to the number of positive tumor cells (low, score 0-2, <50% tumor cells positive and high, score 3 and 4, >50% tumor cell positive). Fisher's exact test was used to compare low and high positive cases according to sex (male vs. female), MSI status (MSS vs. MSI$^+$) and TP53 and KRAS (wild-type vs. mutated). No statistical significance was found between groups (data not shown).

Antiviral GCV and/or anti-COX-2 CCX therapy decreases viral transcript expression in HCMV-infected CRC cells. Caco-2 and LS-174T cells were infected *in vitro* with the HCMV strain VR1814, harvested at 1, 3, 7 dpi and subjected to RT-qPCR for HCMV IE and pp65 transcript analysis. Mock-infected cells did not express any viral transcripts, while HCMV IE transcripts were detected at 3 and 7 dpi in infected Caco-2 cells (Fig. 3B and C) and 1-7 dpi in infected LS174T

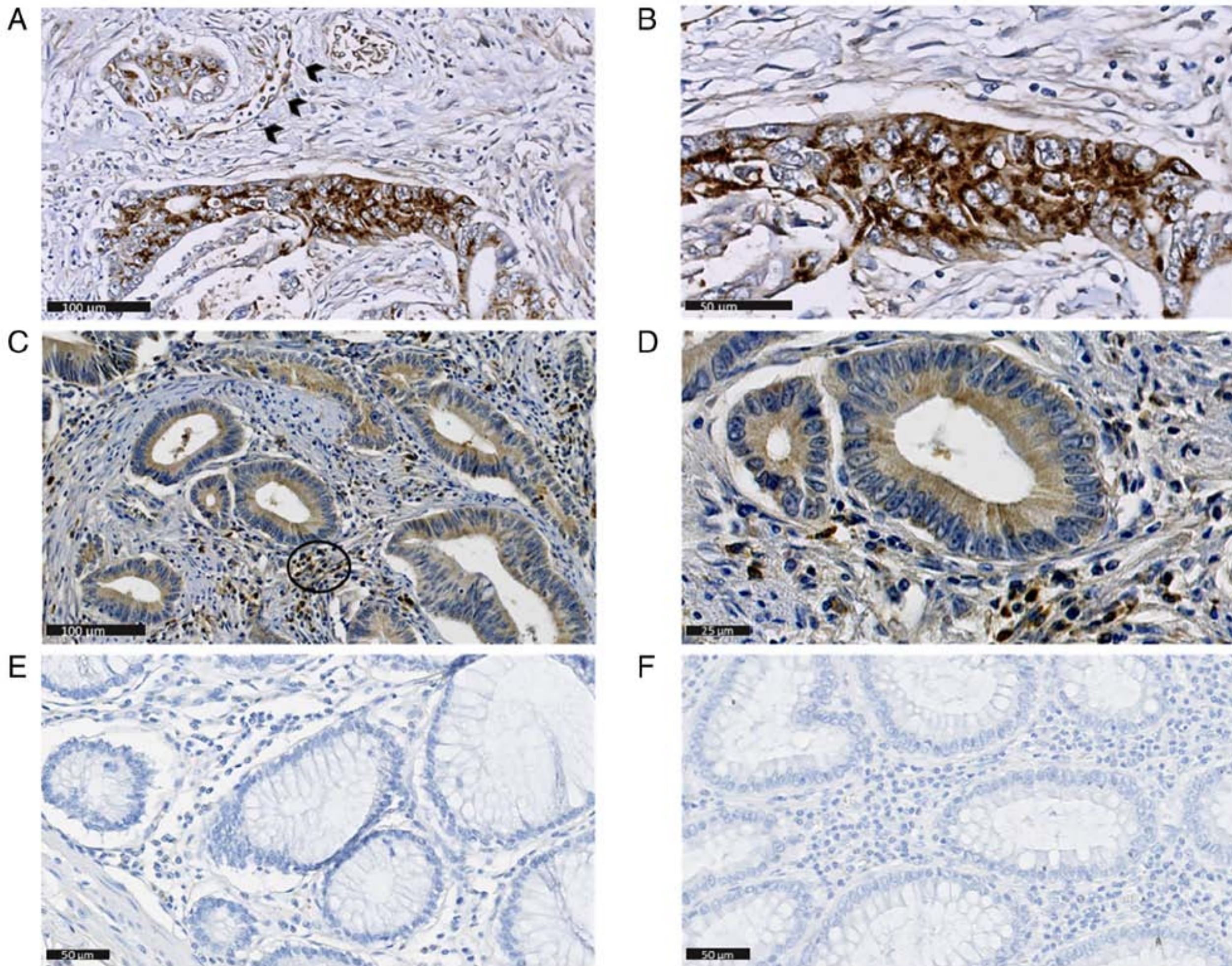

Figure 1. Representative micrographs of immunostaining for human Cytomegalovirus IE and late protein pp65 in CRC and normal colorectal mucosa. (A) IE is expressed in the cytoplasm of cancer cells in a punctate pattern and as indicated by the arrows in tumor associated vessels´ endothelial cells; (B) magnification. (C) Pp65 shows a diffuse cytoplasmic immunostaining evident in colorectal cancer cells and as indicated by the cirle in tumor-associated interstitial cells and macrophages; (D) magnification of C. (E) IE immunostaining is not found in non-tumorous mucosa. (F) Pp65 immunostaining is not found in non-tumorous mucosa. IE, immediate early; CRC, colorectal cancer.

cells (Fig. 3E-G). The late transcript pp65 was detected in both cell lines but only at 7 dpi (Fig. 3D and H), indicating slow or defective HCMV replicaton cycle in colon cancer cells. To assess whether anti-viral or COX-2 inhibitory drugs affected HCMV replication, infected cells were treated with GCV at 100 μM and/or CCX at 10 μM. GCV and/or CCX significantly decreased IE and pp65 transcript levels in both infected cell lines at all time points except CCX in LS-174T at 1 and 3 dpi and GCV in LS-174T at 3 dpi. The combination of drugs was more effective in decreasing HCMV IE transcripts in LS-174T cells at 1 dpi compared with GCV or CCX alone.

HCMV induces COX-2 and 5-LO expression in CRC cells. To assess whether HCMV could activate pro-inflammatory pathways, COX-2 and 5-LO transcripts were analyzed by RT-qPCR at 1, 3 and 7 dpi in VR1814-infected Caco-2 and LS-174T cells (Fig. 4). In Caco-2 cells, HCMV significantly induced COX-2 expression at 1, 3 and 7 dpi compared with mock-infected cells. VR1814-infected Caco-2 cells treated with GCV and/or CCX had lower COX-2 transcript levels compared with untreated infected cells (Fig. 4A-C). Likewise, in LS-174T cells, HCMV VR1814 infection significantly induced COX-2 expression at 1, 3 and 7 dpi compared with mock-infected cells (Fig. 4D-F). LS-174T cells treated with GCV and/or CCX showed a significant decrease in COX-2 transcript levels compared with untreated infected cells at all time point.

5-LO transcripts were analyzed by RT-qPCR at 1, 3 and 7 dpi in mock- and HCMV-infected Caco-2 and LS-174T cells (Fig. 5). In Caco-2 cells, HCMV infection significantly upregulated 5-LO expression at 3 and 7 dpi compared with mock-infected cells but not at 1 dpi. Infected Caco-2 cells treated with GCV showed decreased 5-LO transcript levels at 3 and 7 but not at 1 dpi This was coherent with the fact that the HCMV-infected cells did not reveal increased 5-LO transcript levels at 1 dpi compared with mock-infected cells and confirms specificity of GCV antiviral action. CCX and CCX + GCV downregulated 5-LO levels at all time points in infected Caco-2 cells. CCX was more effective than GCV in reducing 5-LO transcripts at 7 dpi. Similarly, in LS-174T cells, HCMV infection significantly induced 5-LO transcript expression at 1, 3 and 7 dpi compared with mock-infected cells. Compared with untreated infected cells, infected LS-174T cells treated with GCV, CCX or GCV + CCX had significantly lower levels of 5-LO transcripts at each time point. GCV + CCX was

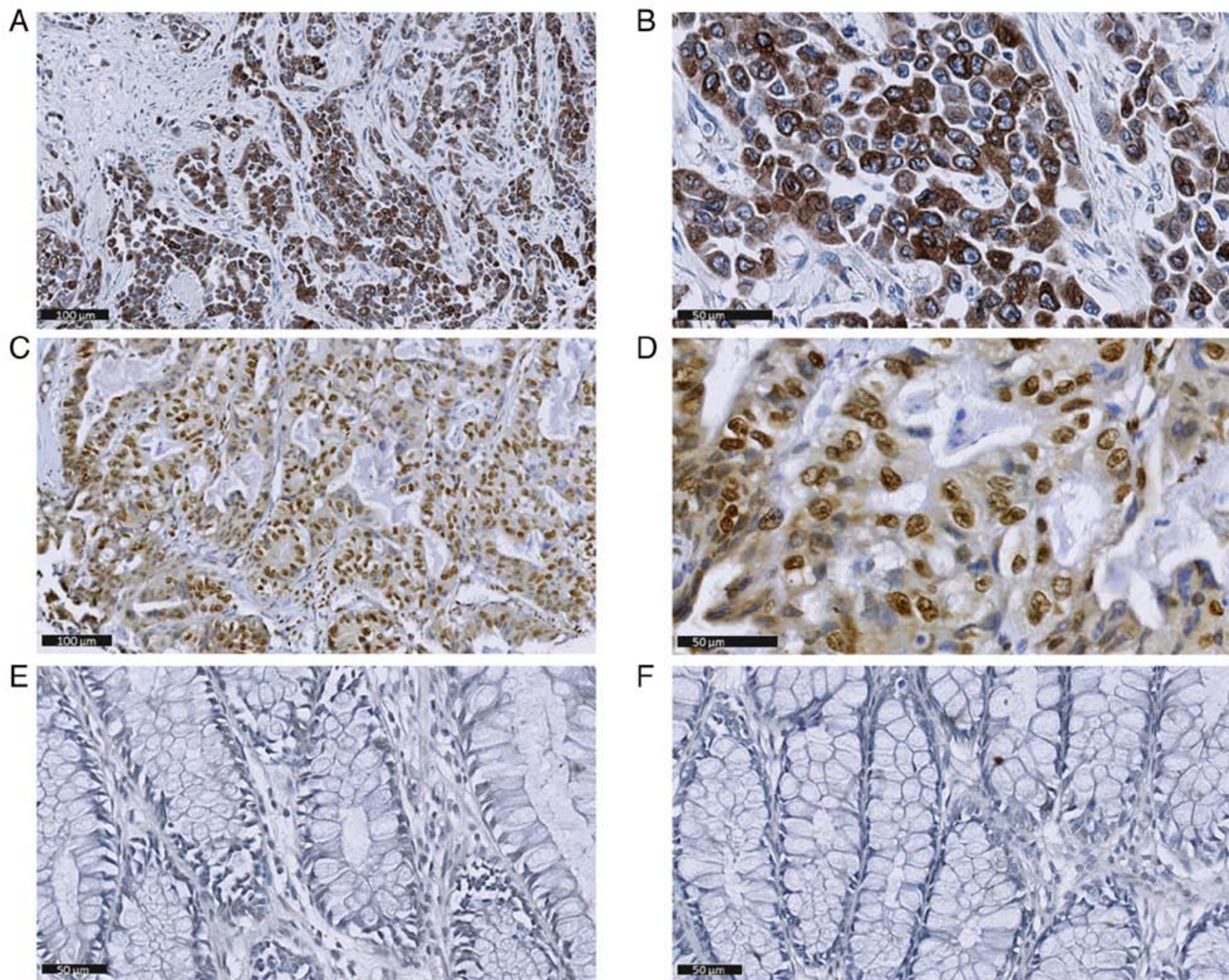

Figure 2. Representative micrographs of COX-2 and 5-LO immunostaining in colorectal cancer and non-tumorous colorectal mucosa. (A) Cytoplasmic COX-2 is strongly expressed in colorectal cancer cells; (B) magnification of A. (C) Primarily nuclear 5-LOis strongly and diffusely expressed in colorectal cancer cells. (D) magnification of D. (E) COX-2 is not expressed in non-tumorous crypt cells. (F) 5-LO is not expressed in non-tumorous crypt cells (Scale bar, 50 μm. COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase.

more effective in reducing 5-LO transcript expression than GCV or CCX alone at 1 and 3 dpi. At 7 dpi, CCX alone or in combination with GCV was more effective than GCV alone.

HCMV induces expression of the inflammatory proteins 5-LO and COX-2 in CRC cells. Caco-2 and LS-174T cells were plated onto chamber slides and infected with HCMV strain VR1814 at MOI 5 or mock-infected. At 7 dpi the cells were fixed and stained for IE, COX-2 and 5-LO and subsequently analyzed by IF. Infected cells exhibited higher expression of both COX-2 and 5-LO compared with mock-infected cells (Fig. 6).

HCMV induces proliferation of CRC cells. To assess whether HCMV infection altered the proliferation rate of colon cancer cells, MTT assay was performed to assess proliferation in mock- and HCMV-infected CRC cell lines. HCMV-infected CRC cells had a higher proliferation than mock-infected cells at late time points (Fig. 7). HCMV-infected Caco-2 cells showed higher proliferation capacity at 7 dpi (Fig. 7C). HCMV-infected LS-174T cells showed higher proliferative capacity compared with mock-infected cells at 3 and 7 dpi (Fig. 7E and F). No significant difference in proliferation activity was observed at 1 or 3 dpi in Caco-2 cells (Fig. 7A and B) or at 1 dpi in LS-174T (Fig. 7D).

HCMV-infected CRC cells do not produce infective viral particles. The present study tested the effect of antiviral therapy with GCV and COX-2 inhibitor CCX on production of infectious virus particles in Caco-2 and LS-174T cells. No IE-positive staining was observed by IF in MRC5 cells incubated with supernatant from infected Caco-2 and LS-174T cells (data not shown), thus no evidence of virions produced in these colorectal cancer cells was found at 7 dpi.

Discussion

The presence of HCMV in cancer has been extensively studied (37,71) but there is no consensus regarding the clinical relevance of these observations. Recently, Peredo-Harvey *et al* (72) published a systematic review on diagnostic methods for detection of HCMV in glioblastoma and concluded that the discordant data reflect unresolved technical issues as optimized techniques to detect HCMV nucleic acids and proteins in tumors have not been developed (72-74).

Table II. Spearman correlation analysis of viral and inflammatory proteins and immunohistopathological and clinical variables.

A, IE

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	144	142	143	144	140	143	116	116	116
ρ	1	0.3487	0.4347	0.5415	0.2958	-0.0672	0.0334	0.0489	0.0656
P	NA	<0.0001	<0.0001	<0.0001	0.0004	0.4249	0.7219	0.6022	0.4839

B, pp65

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	142	142	141	142	138	141	114	114	114
ρ	0.3487	1	0.1706	0.3200	0.2913	0.0571	0.0530	-0.1238	0.0444
P	<0.0001	NA	0.0432	0.0001	0.0005	0.5013	0.5751	0.1893	0.6391

C, 5-LO

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	143	141	145	143	140	144	118	118	118
ρ	0.4347	0.1706	1	0.3573	0.1553	0.0378	-0.1273	-0.1070	-0.0055
P	<0.0001	0.0432	NA	<0.0001	0.0669	0.6529	0.1695	0.2487	0.9529

D, COX-2

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	144	142	143	144	140	143	116	116	116
ρ	0.5415	0.3200	0.3573	1	0.2311	-0.0784	-0.0164	-0.0298	0.1486
P	<0.0001	0.0001	<0.0001	NA	0.0060	0.3519	0.8610	0.7504	0.1114

E, Ki-67

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	140	138	140	140	140	140	114	114	114
ρ	0.2958	0.2913	0.1553	0.2311	1	0.1284	-0.0732	-0.0899	-0.0362
P	0.0004	0.0005	0.0669	0.0060	NA	0.1307	0.4387	0.3417	0.7019

F, EGFR

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
n	143	141	144	143	140	145	118	118	118
ρ	-0.0672	0.0571	0.0378	-0.0784	0.1284	1	-0.0383	-0.0402	0.0465
P	0.4249	0.5013	0.6529	0.3519	0.1307	NA	0.6804	0.6659	0.6170

G, Differentiation grade

n	116	114	118	116	114	118	118	118	118

Table II. Continued.

Value	IE	pp65	5-LO	COX-2	Ki-67	EGFR	Differentiation grade	Duke's stage	Age
G, Differentiation grade									
ρ	0.0334	0.0530	-0.1273	-0.0164	-0.0732	-0.0383	1	-0.0267	-0.1837
P	0.7219	0.5751	0.1695	0.8610	0.4387	0.6804	NA	0.7742	0.0465
H, Duke´s stage									
n	116	114	118	116	114	118	118	118	118
ρ	0.0489	-0.1238	-0.1070	-0.0298	-0.0899	-0.0402	-0.0267	1	-0.0027
P	0.6022	0.1893	0.2487	0.7504	0.3417	0.6659	0.7742	NA	0.9771
I, Age									
n	118	114	118	116	114	118	118	118	118
ρ	-0.005	0.0444	-0.0055	0.1486	-0.0362	0.0465	-0.1837	-0.0027	1
P	0.9529	0.6391	0.9529	0.1114	0.7019	0.6170	0.0465	0.9771	NA

IE, immediate early; pp65, posphoprotein 65; 5-LO, 5-lipoxygenase; COX-2, cyclooxygenase-2; NA, not applicable.

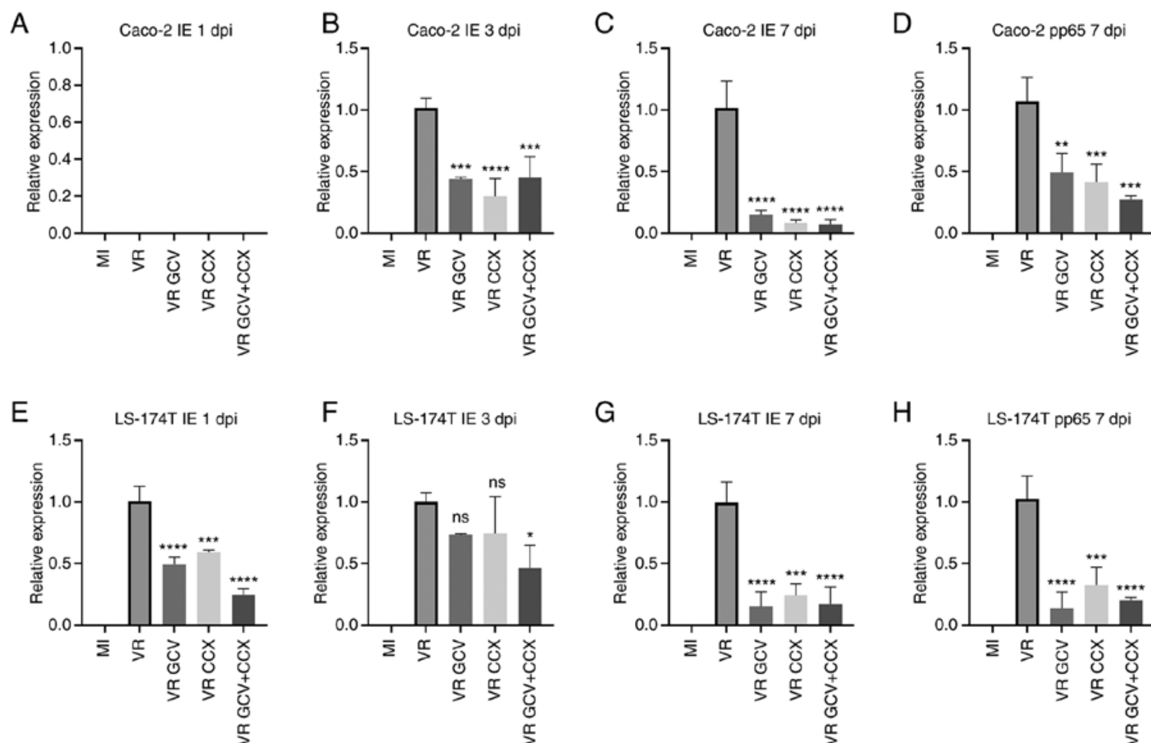

Figure 3. HCMV IE and pp65 transcript expression in HCMV-infected Caco-2 and LS-174T cells is reduced by GCV and CCX. Relative IE and pp65 expression levels were determined by reverse transcription-quantitative PCR. The bars represent COX-2 relative expression to the housekeeping gene. (A) No IE transcripts were detected at 1 dpi in HCM-infected or mock-infected Caco-2 cells; (B) IE transcripts were detected at 3 dpi in HCMV infected Caco-2 cells (VR) and they were reduced by treamtents with GCV and CCX. (C) IE transcripts were detected at 7 dpi in VR) and they were reduced by treamtent with GCV and CCX. (D) Pp65 transcripts were detected at 7 pi in HCMV-infected Caco-2 cells and reduced by GCV and CCX treatments. Only the 7 dpi timepoint is shown for pp65 since no transcripts were detected at earlier timepoints (E) No IE transcripts were detected at 1 dpi in HCM-infected or mock-infected LS-174T cells; (F) IE transcripts were detected at 3 dpi in HCMV infected LS-174T cells (VR) and they were reduced by treamtents with GCV and CCX; (G) IE transcripts were detected at 7 dpi in HCMV infected LS-174T cells (VR) and they were reduced by treamtents with GCV and CCX; (H) Pp65 transcripts were detected at 7 pi in HCMV-infected LS-174T cells and reduced by GCV and CCX treatments. Only the 7 dpi timepoint is shown for pp65 since no transcripts were detected at earlier timepoints. Data are presented as the mean ± SD. Statistical significance was determined by ANOVA test. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001 vs. VR. HCMV, human cytomegalovirus; IE, immediate early; pp65, posphoprotein 65; GCV, ganciclovir; CCX, celecoxib; MI, mock-infected; VR, virus-infected; dpi, days post infection; ns, not significant.

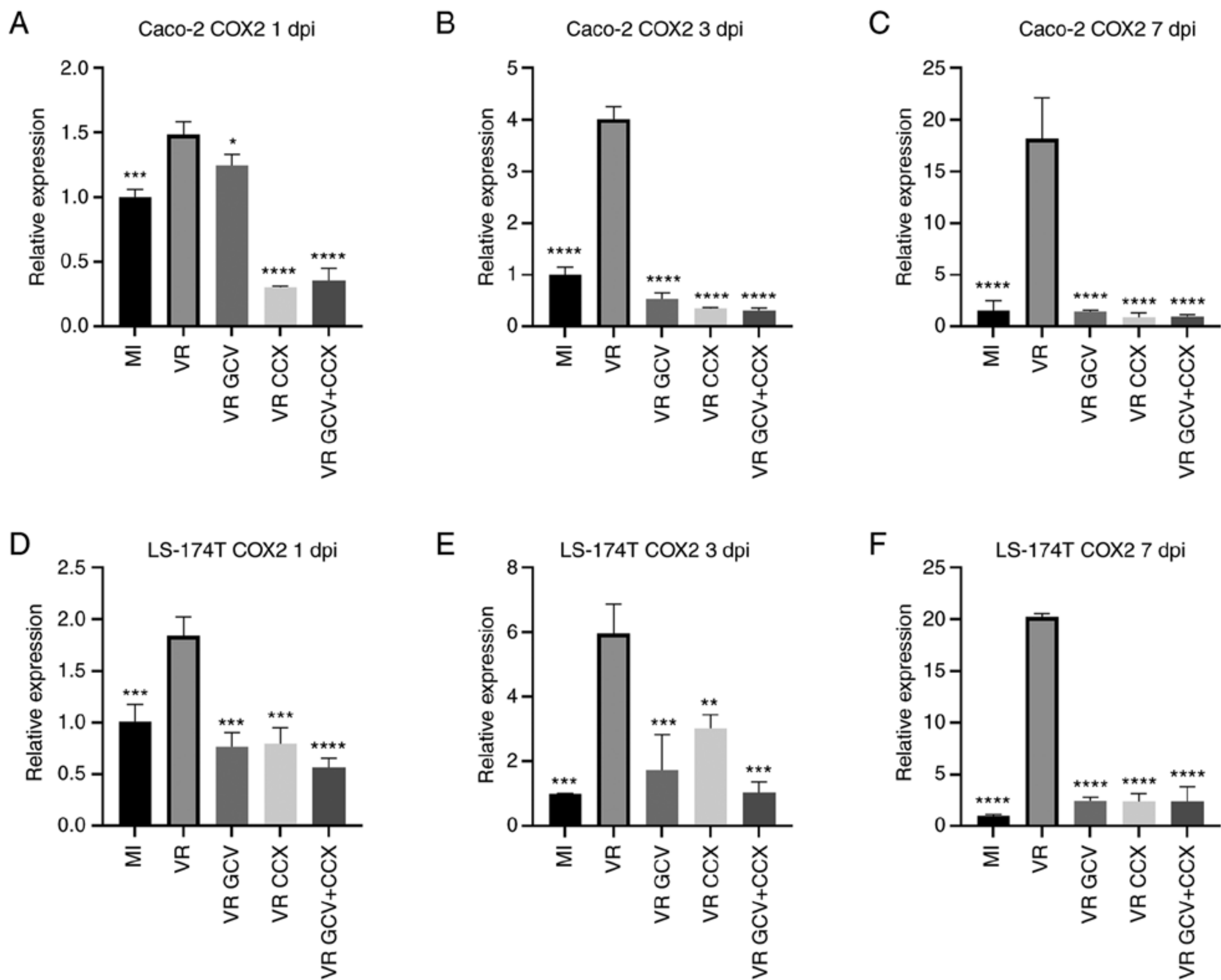

Figure 4. HCMV-infected Caco-2 and LS-174T cells exhibit increased COX-2 expression. Relative COX-2 expression was determined by reverse transcription-quantitative PCR at 1, 3 and 7 dpi. Data are presented as the mean ± SD. Caco-2 infected with HCMV (VR) shows higher COX-2 transcripts than mock-infected cells (MI) at 1 dpi (A), 3 dpi (B) and 7 dpi (C). Treatment with GCV, CCX or a combination of the two drugs significantly reduced COX-2 transcripts at all time points in Caco-2 HCMV-infected cells. LS-174 cells infected with HCMV (VR) shows higher COX-2 transcripts than mock-infected cells (MI) at 1 dpi (D), 3 dpi (E) and 7 dpi (F). Treatment with GCV, CCX or a combination of the two drugs significantly reduced COX-2 transcripts at all time points in LS-174 HCMV-infected cells. Statistical significance was determined by ANOVA test. Statistical significance was determined by ANOVAtest. $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$ vs. VR. HCMV, human cytomegalovirus; COX-2, cyclooxygenase-2; GCV, ganciclovir; CCX, celecoxib; MI, mock-infected; VR, virus-infected; dpi, days post infection.

The high expression of HCMV in tumors may serve a key role in tumorigenesis and tumor progression as this virus can cause all ten hallmarks of cancer (75,76), promote tumor cell proliferation and serve as a target for therapy.

Taher *et al* (54) reported that HCMV DNA, transcripts and protein are present not only in primary CRC but also in brain metastases of patients with CRC and that high grade of HCMV infection in primary tumors is associated with shorter time to development of brain metastases and shorter overall survival time. HCMV protein expression is confined to tumor cells and metastases and is not present in healthy surrounding tissue. Harkins *et al* (18) reported that HCMV IE72 and COX-2 are co-expressed in CRC and HCMV infection *in vitro* induces COX-2 expression in Caco-2 cells. HCMV DNA and proteins are rarely found in non-tumorous tissue surrounding primary CRC or metastases, which suggests a tumor-specific connection and hence potential role of this virus in CRC (18). The present study examined 146 Iranian CRC cases, of which 144 were analyzed by IHC for IE and pp65, key HCMV proteins representative of IE and late phase of viral replication. Both viral proteins were diffusely expressed in most of cases, consistent with previous studies (18,56). The association between levels of immunostaining for HCMV proteins, immunohistochemical expression of COX-2, 5-LO, Ki-67 and EGFR, TP53 and KRAS mutation and MSI status was assessed. HCMV proteins were similarly expressed in tumors regardless of Duke's grades, TP53 and/or KRAS mutation, MSI status and EGFR membrane expression. By contrast, HCMV IE and pp65 expression levels were positively correlated with 5-LO, COX-2 and Ki-67, suggesting that higher HCMV load was associated with more aggressive pro-inflammatory tumor phenotype, regardless of tumor genetic and molecular background.

Inflammation is a hallmark of cancer and is considered to be essential for carcinogenesis. HCMV promotes inflammation (50,77-78); inflammation is linked to HCMV reactivation and replication (79-82). The HCMV major immediate early promoter that controls expression of IE proteins is activated by inflammatory cytokines such as TNF-α and IL-6, which induce

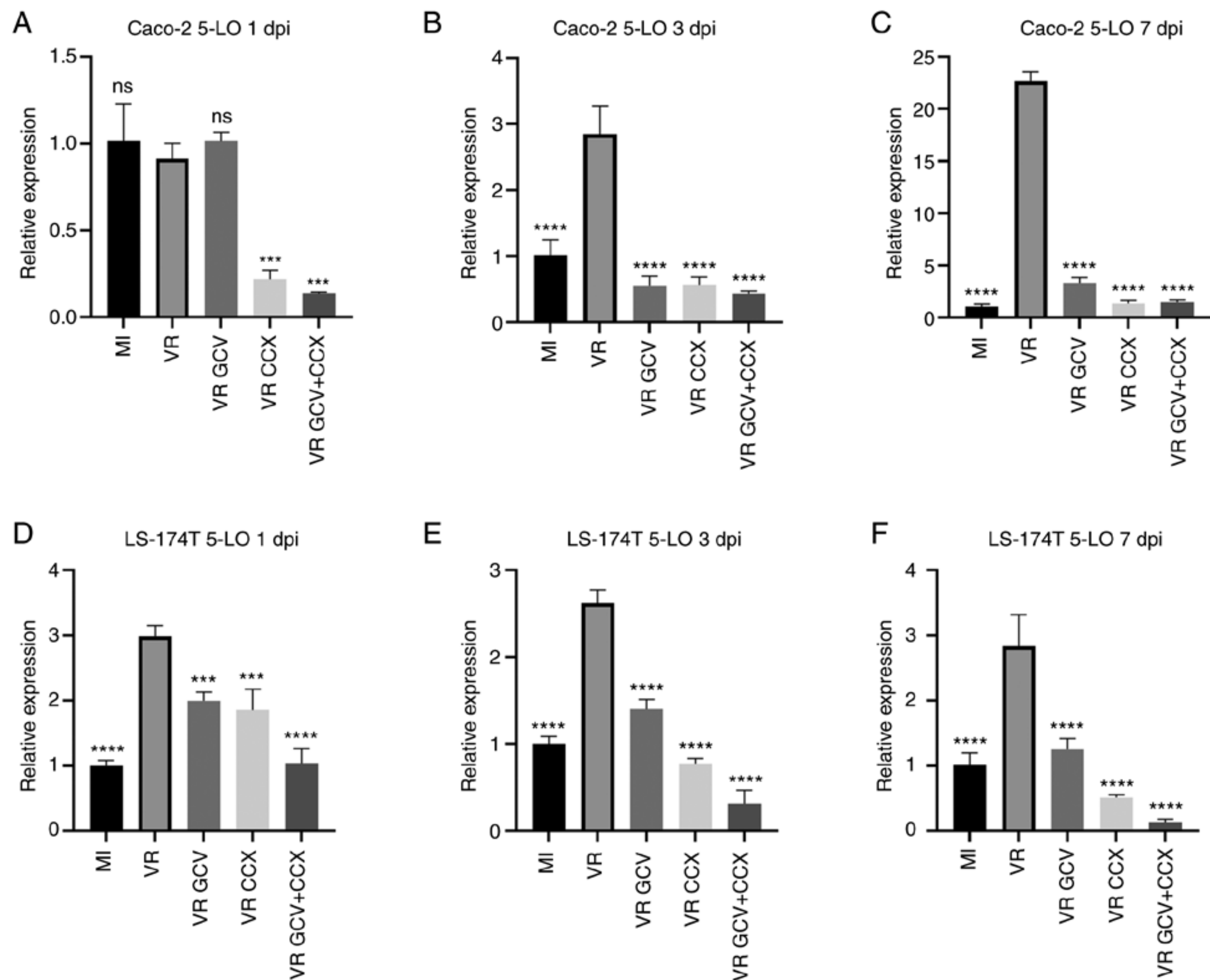

Figure 5. HCMV increases 5-LO expression in Caco-2 and LS-174T cells. Relative 5-LO expression was determined by qPCR at 1, 3 and 7 dpi. The bars represent 5-LO relative expression to the housekeeping gene. Data are presented as the mean ± SD. (A) At 1 dpi Caco-2 infected with HCMV (VR) do not show higher 5-LO transcripts than MI); GCV treatment on infected cells did not decrease 5-LO transcripts at 1 dpi, on the contrary CCX and a combination of GCV and CCX reduce 5-LO transcripts in HCMV-infected cells. At 3 dpi (B) and 7 dpi (C) Caco-2 infected with HCMV (VR) shows higher 5-LO transcripts than mock-infected cells (MI). Treatment with GCV, CCX or a combination of the two drugs significantly reduced 5-LO transcripts at 3 dpi (B) and 7 dpi (C) in Caco-2 HCMV-infected cells. LS-174 cells infected with HCMV (VR) shows higher 5-LO transcripts than mock-infected cells (MI) at 1 dpi (D), 3 dpi (E) and 7 dpi (F). Treatment with GCV, CCX or a combination of the two drugs significantly reduced 5-LO transcripts at all time points in LS-174 HCMV-infected cells. Statistical significance was determined by ANOVA test. ***$P\leq 0.001$, ****$P\leq 0.0001$ vs. VR. HCMV, human cytomegalovirus; 5-LO, 5-lipoxygenase; GCV, ganciclovir; CCX, celecoxib; MI, mock-infected; VR, virus-infected; dpi, days post infection; ns, not significant.

reactivation of latent HCMV in myeloid lineage cells (79,83). HCMV also induces expression of 5-LO and COX-2 and the production of PGE2 and LTB4, which are potent inflammatory mediators that attract inflammatory cells to sites of infection (51,77,78,84); this promotes inflammation that can drive HCMV replication. The viral chemokine receptor homologue US28 induces STAT3 expression and IL-6 production and IL-1β is produced via activation of absent in melanoma 2 inflammasomes (85). HCMV infection induces production of pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, IL-17, TNF-α granulocyte colony-stimulating factor (CSF), granulocyte-macrophage CSF and chemokines such as monocyte chemoattractant protein, macrophage inflammatory protein-1 and IFN-inducing protein 10 (86).

In the present study, both 5-LO and COX-2 were found to be expressed in HCMV-infected CRC specimens and were associated with higher Ki-67 index. HCMV induces cell proliferation and increase tumor aggressiveness (87), which may reflect its strong connection with inflammatory signaling. HCMV-encoded tumorigenic chemokine receptor homologue US28 (88,89) directly induces COX-2 expression (50). Transgenic mice expressing US28 in intestinal epithelial cells develop intestinal adenomas and adenocarcinoma by 40 weeks of age, a process that is significantly enhanced by a combined treatment with azoxymethane and dextran sodium sulfate, which promote inflammation (59).

Our previous study reported that HCMV contributes to inflammation in breast cancer and medulloblastoma, potentially by driving COX-2 and/or 5-LO expression (45,53). HCMV DNA and proteins co-localize with inflammatory mediator IL-6 (90) and HCMV-infected vascular smooth muscle cells express high levels of 5-LO protein in tissue samples from patients with inflammatory bowel disease (77), which suggests a broader role of HCMV in the intestine. The present study demonstrated that HCMV infection induced COX-2 and 5-LO expression and proliferation in Caco-2 cells, which maintains

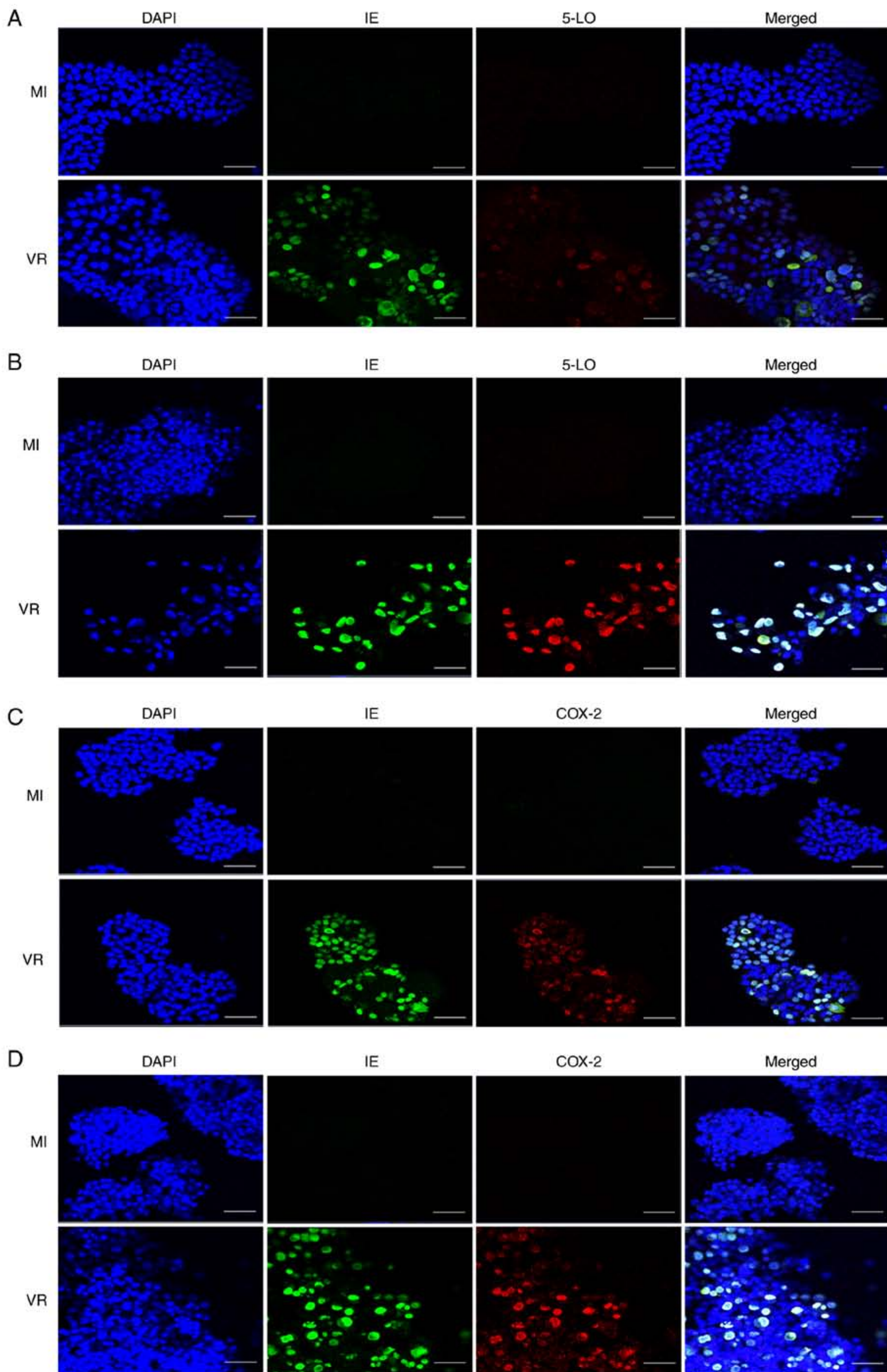

Figure 6. Immunofluorescence analysis of 5-LO and COX-2 protein staining in human cytomegalovirus (HCMV) infected colorectal cancer cells. Caco-2 and LS-174T cells at 7 days post-infection were stained for of IE (green) and 5-LO or COX-2 (red). IE was detectable only in infected cells. 5-LO was upregulated in infected (A) Caco-2 and (B) LS-174T cells. COX-2 was upregulated in infected (C) Caco-2 and (D) LS-174T cells. Scale bar, 100 μm. 5-LO, 5-lipoxygenase; COX-2, cyclooxygenase-2; IE, immediate early; MI, mock-infected; VR, virus-infected.

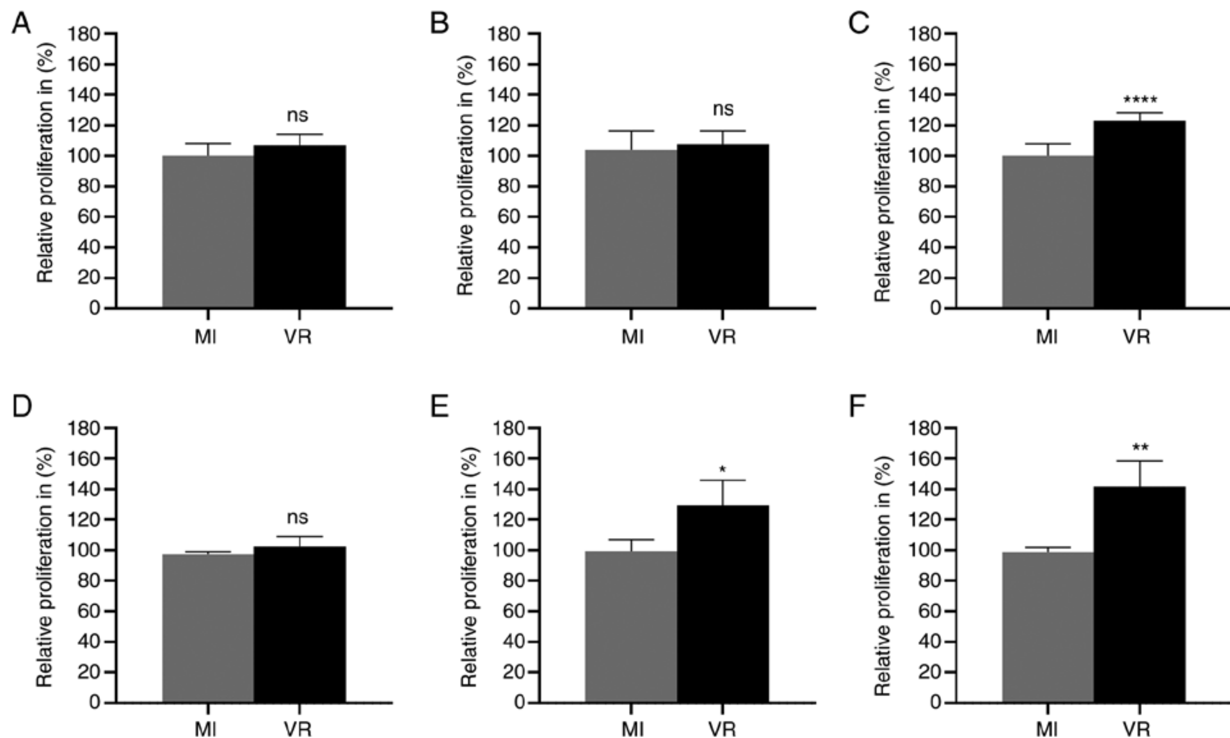

Figure 7. Increased proliferation in VR. Proliferation of HCMV-infected colorectal cancer cells (VR was analyzed by MTT assay and values were normalized to mock-infected cells (MI). Data are presented as the mean ± SD. *P≤0.05, **P≤0.01, ****P≤0.0001 vs. MI. VR) do not show a higher proliferation rate than MI cells at 1 dpi (A) or at 3 (dpi). HCMV-infected Caco-2 cells VR) show a higher proliferation rate than MI cells at (B) 3 dpi (B) and at 7 dpi (C). HCMV-infected LS-174T cells (VR) do not show a higher proliferation rate than MI cells at 1 dpi (D) or at 3 dpi (E). HCMV-infected LS-174T cells (VR) show a higher proliferation rate than MI cells at 7 dpi (F). HCMV, human cytomegalovirus; dpi, days post-infection; ns, not significant; MI, mock-infected; VR,virus-infected.

enterocytic differentiation potential, and LS-174, an aggressive, poorly differentiated cell line (65). Caco-2 cells have similar behavior to normal differentiated villus cells and are well-characterized in the Wnt pathway and its role in confluency and cancer (91,92). The fact that HCMV induces inflammation and proliferation in both Caco-2 and LS-174 cells strengthen the potential role of HCMV as inflammation occurs in the normal tissues and is critical in the process of CRC development (11-13). Here, anti-viral therapy with GCV effectively reduced virus-induced COX-2 and 5-LO transcript expression in both Caco-2 and LS-174T cells. Similarly, COX-2 inhibitor CCX decreased expression of COX-2 and 5-LO as well as HCMV IE and pp65 transcripts, suggesting a mutual loop. VGCV, a prodrug of GCV, significantly decreases growth of HCMV-positive medulloblastoma xenografts in mice and combination with CCX results in a significant additive effect, decreasing tumor growth by 72% (45). HCMV benefits from a pro-inflammatory environment and anti-inflammatory drugs effectively decrease replication in infected cells (51,93,94) and tumor cell proliferation

Viral infectivity assay showed that no infectious virions were produced in HCMV infected Caco-2 and LS-174 cells, consistent with previous literature reporting that HCMV replicates poorly or not at all in cancer cells (95). This may be dependent on the virus inability to arrest cells in G1/G2 phase, which is important for to initiate DNA replication. There was no evidence of virions produced in supernatant of these colon cancer cells at 7 dpi, indicating that the effects of HCMV on colon cancer cells may be associated with virus ability to express proteins and thereby affect cell function (95).

HCMV is not able to fully replicate in cancer cells may be one of the driving forces of oncomodulation as, for example, HCMV infection fuels genomic instability in non-permissive cells such as cancer cells (95).

Future studies should investigate how HCMV controls the cell cycle in normal and cancer cells, how COX-2 and 5-LO expression is regulated in different cancer cells and the mechanisms that HCMV uses to induce their expression in cancer cells.

Taken together with literature (18,41,56), the present results confirmed that HCMV was present in CRC, regardless of stage and molecular/genetic background, with higher viral activity associated with an aggressive pro-inflammatory phenotype, and suggested that anti-viral drugs, alone or in combination with anti-inflammatory treatment, could be effective in patients with CRC. Here, HCMV infection resulted in an upregulation of COX-2 and 5-LO in CRC cells. These inflammatory mediators promote tumor aggressiveness and hence therapies that interfere with their release or effects on neighboring tumor cells may affect tumor growth. Aspirin reduces risk of colon cancer and metastastic disease (6). Aspirin, initially given to decrease platelet formation and risk of reinfarction in patients with myocardial infarction, significantly decreases cancer risk (96). Other anti-platelet therapy that interferes with production and release of AA may exert negative downstream effects on cancer cells (97,98). Examples of these include the P2Y12 inhibitor tricagrelor that inhibits tumor-platelet interaction and metastasis formation (99), as well as clopidogrel (100) and flavonoids with anti-COX and anti-cancer effects (101,102).

Limitations of the present study include the lack of clinical data such as therapy, recurrence and survival. Moreover, the present *in vitro* findings need to be validated in animal models before possible investigation of the potential therapeutic effect of antiviral drugs, alone or in combination with anti-inflammatory agents in patients with CRC. Further studies need to assess the impact of anti-inflammatory and anti-viral drugs in preventing malignant transformation, tumor proliferation and metastasis formation.

The efficacy of antiviral therapy against HCMV has been already addressed in patients with newly diagnosed, recurrent and secondary glioblastoma, where VGCV given as add on to standard therapy shows good tolerability and significantly improved survival (103,104,105). Dendritic cell vaccination with HCMV pp65 mRNA also improves survival rates among patients with glioblastoma (106,107). Both strategies are currently being evaluated in randomized phase II trials (trial nos. NCT04116411 and NCT00639639), which could provide proof of concept for the use of HCMV-targeted therapies in patients with glioblastoma and other HCMV-positive tumors, such as CRC. This may provide potential novel therapeutic options for patients with HCMV-positive cancer, including combined therapy with drugs targeting both the virus and COX-2/5-LO pathways.

In conclusion, the present study detected high levels of HCMV IE, pp65, COX-2 and 5-LO in most CRC samples but not in non-malignant mucosa. There was a significant correlation between higher expression of HCMV proteins (IE and pp65) and higher COX-2 and 5-LO protein levels. Ki-67 index was correlated with higher load of viral proteins, suggesting that HCMV infection was associated with more aggressive, proliferative tumor phenotype. The *in vitro* results demonstrated that HCMV induced expression of COX-2 and 5-LO in CRC cell lines that represent different tumor phenotypes. Antiviral therapy with GCV effectively reduced transcript levels of HCMV genes encoding IE and pp65 and the cellular genes encoding COX-2 and 5-LO. The anti-inflammatory drug CCX decreased transcript levels of 5-LO and COX-2 as well as HCMV IE and pp65 viral transcripts. A combination of GCV and CCX was more effective than monotherapy. These results provide a basis for mechanistic studies *in vitro* and preclinical *in vivo* validation. Combined anti-HCMV and anti-inflammatory agents may serve as a novel therapeutic options for patients with CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MRP, AR, RMC, CSN and CT conceived the study. MRP, CSN and RMC wrote and edited the manuscript. MRP, NMA, RL, MM, SV, CT and SDF performed experiments. RMC, RL, AR, and MRP analyzed the data. FV, FB and MM edited the manuscript and colleted the data. RMC, AR and CSN supervised the study. MRP and NMA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed according to the Declaration of Helsinki for human medical research and approved by the ethics committee of the Digestive Disease Research Center of Tehran University of Medical Sciences (approval no. #91033718830). The requirement for patient consent was waived as samples were anonymized and had been stored for ~20 years.

Ethics approval for the non-tumorous colorectal mucosa samples, previously collected at the Department of Molecular Medicine and Surgery at Karolinska Institutet, was obtained from Regional Human Ethics Committee, Stockholm, Sweden (No2008/628-31).

Patient consent for publication

Not applicable.

Competing interests

CSN holds a patent on diagnostics and treatment of a CMV variant strain found in cancer (patent no. US9701943B2). The other authors declare that they have no competing interests.

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