Research Article

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Clusterin mRNA silencing reduces cell proliferation, inhibits cell migration, and increases *CCL5* expression in SW480, SW620, and Caco2 cells

Clusterin mRNA susturulması hücre proliferasyonunu azaltır, hücre göçünü engeller ve SW480, SW620 ve Caco2 hücrelerinde *CCL5* ifadesini arttırır

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Abstract

Objectives: This study aimed to investigate the effects of specific gene silencing in colorectal cancer cells. Clusterin protein was found in the serum samples of colorectal cancer patients infected with *Schistosoma mansoni* previously.

Methods: For this reason, silencing clusterin mRNA in colorectal cancer cells was first performed to study the cytotoxic effect by lactate dehydrogenase assay. Next, propidium iodide staining and flow cytometry were performed to investigate the cell cycle profile in clusterin-silenced cells. A wound-healing assay was also used to examine the migration rate of clusterin-silenced cells. The mRNA expression of cell proliferation- and migration-related genes was then assessed by real-time PCR.

Results: Clusterin mRNA silencing caused a significant reduction in cell growth but induced no cell cycle arrest or potential apoptosis in all cells. It was found in this study that cell migration rate was inhibited in clusterin-silenced cells. Surprisingly, significantly induced chemokine (C–C motif) ligand 5 (CCL5) mRNA expression was detected in clusterin-silenced Caco2, which indicated that the cell proliferation and migration of clusterin-silenced Caco2 were likely associated with CCL5 mRNA expression.

Conclusions: Clusterin may be a potential target for regulation, staging, surveillance, and developing a cost-effective therapeutic agent for treating parasite-infected Caco2 type of colorectal cancer patients.

Keywords: CCL5 mRNA expression; clusterin mRNA silencing; colorectal cancer cells; migration inhibition; proliferation reduction.

ÖZ

Amaç: Bu çalışmada kolorektal kanser hücrelerinde spesifik gen susturmanın etkilerinin araştırılması amaçlandı. Daha önce, *Schistosoma mansoni* ile enfekte kolorektal

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kanser hastalarının serum örneklerinde Clusterin proteini bulunmuştu.

Yöntemler: Bu nedenle, laktat dehidrojenaz testi ile sitotoksik etkiyi incelemek için ilk olarak kolorektal kanser hücrelerinde clusterin mRNA'sının susturulması gerçekleştirildi. Daha sonra, clusterin'i susturulmuş hücrelerde hücre döngüsü profilini araştırmak için propidium iyodür boyama ve akış sitometrisi yapıldı. Ayrıca, clusterin'i susturulmuş hücrelerin göç hızını incelemek için yara iyileştirme analizi kullanıldı. Daha sonra, hücre proliferasyonu ve migrasyonla ilgili genlerin mRNA ekspresyonu gerçek zamanlı PCR ile değerlendirildi.

Bulgular: Clusterin mRNA susturma, hücre büyümesinde önemli bir azalmaya neden oldu, ancak tüm hücrelerde hücre döngüsü durmasına veya potansiyel apoptoza neden olmadı. Bu çalışmada clusterin'i susturulmuş hücrelerde hücre göç hızının engellendiği bulundu. Şaşırtıcı bir şekilde, clusterin'i susturulmuş Caco2'de önemli ölçüde indüklenmiş kemokin (C-C motifi) ligandı 5 (CCL5) mRNA ifadesi tespit edildi; bu, clusterin'i susturulmuş Caco2'nin hücre çoğalmasının ve göçünün muhtemelen CCL5 mRNA ifadesi ile ilişkili olduğunu gösterdi.

Sonuçlar: Clusterin, parazitle enfekte Caco2 tipi kolorektal kanser hastalarını tedavi etmek için düzenleme, evreleme, gözetim ve uygun maliyetli bir terapötik ajan geliştirmek için potansiyel bir hedef olabilir.

Anahtar Kelimeler: CCL5 mRNA ekspresyonu; clusterin mRNA susturma; kolorektal kanser hücreleri; migrasyon inhibisyonu; proliferasyon azalması.

Introduction

Clusterin is a glycoprotein associated with heterodimeric disulfides secreted in two major isoforms, secretory and intracellular clusterin, with very different functions [1]. These proteins appear to play an important role in cell survival and death [2]. Clusterin protein has been reported to be involved in numerous physiological processes important for carcinogenesis and tumour growth. The processes include apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodelling, lipid transportation, membrane recycling, and immune system regulation [3, 4]. A previous study demonstrated that clusterin protein expression participates in the tumourigenesis and progression of 25% of colorectal cancer, but 75% of tumour specimens comprise cancer cells without expressing clusterin [5, 6]. The clusterin protein expression profile correlation with other cell events indicates that clusterin protein presents only in colorectal cancer patients infected with certain infectious diseases. This phenomenon highlights the potential of clusterin protein to be used as a biomarker in an effective treatment solution for colorectal cancer patients infected with infectious diseases, e.g., Schistosoma. Furthermore, clusterin protein appears to be a sensitive and stable histological marker for murine and human intestinal tumours. The high secretion of clusterin protein from tumour cells can easily be detected in body fluids, e.g., serum. Therefore, clusterin protein can be used as a potential biomarker or potential target for human colorectal cancer treatment.

Our previous study found that patients diagnosed with colorectal cancer and *Schistosoma mansoni* infection secreted a high level of clusterin protein that was not found in the serum of patients diagnosed with colorectal cancer only or infected with *S. mansoni* only [7]. Thus, the present study aimed to determine the role of clusterin using colorectal cancer as a model by performing specific gene silencing in the cells using small interference RNA technology (siRNA). The cytotoxic level in clusterin-silenced cells was first assessed by lactate dehydrogenase (LDH) assay. LDH assay determines the enzyme release from damaged cells, providing a fast and reliable quantification surrogate marker for cytotoxicity. Cell cycle arrest induction and cell migration inhibition were then determined in the gene-specific silenced cells using a wound-healing assay and flow cytometry, respectively. Finally, the cell proliferation and migration-related gene mRNA levels were also performed using real-time PCR in gene-specific silenced cells. SW480, SW620 and Caco2 were used because the cancer cell lines expressed clusterin [8]. Our results pave the way for discovering important factors to be used as biomarkers. These markers, clusterin and CCL5, can also be used to design drugs or cost-effective supplements to effectively treat patients with an infectious disease and colorectal cancer simultaneously in the future.

Materials and methods

Silencing clusterin mRNA in colorectal cancer cells

The analysis used a specific lyophilised Clusterin siRNA Reagent System (Santa Cruz Biotechnology, Inc.) to silence the clusterin mRNA in colorectal adenocarcinoma cell lines (SW480, SW620, and Caco2) as silenced the *IL-21* gene in our previous study [9]. Seeded each cell line at a density of 2×10^5 cells/well in 6 well culture plates. The cells were maintained in 2 mL of antibiotic-free Dulbecco's modified eagle's medium supplemented with 10% foetal bovine serum. The culture was incubated at 37 °C in a 5% (v/v) CO_2 incubator humidified atmosphere

until the cells reached 80% confluence. Solutions A and B were prepared by diluting 4 µL of fluorescein-conjugated clusterin siRNA duplex and 6 µL of siRNA transfection reagent (optimised previously) with 100 µL of siRNA transfection medium, respectively. Solution A was then mixed gently with Solution B and incubated at room temperature for 15–45 min. Then, washed each cell line with 2 mL of siRNA transfection medium. Next, mixed solutions A and B gently with 800 µL of siRNA transfection medium. Overlaid the solutions onto the washed cells prior incubated the transfection reaction at 37 °C for 72 h. The efficacy of specific gene silencing was examined using Western blotting, and the intensity of protein bands was quantitated using ImageJ as described in the previous study [10]. Successful clusterin-silenced cells were then subjected to LDH assay.

Cytotoxic effect analysis of clusterin-silenced colorectal cancer cells

Quantitative cytotoxicity was performed in clusterin-silenced cells using the LDH Cytotoxicity Assay Kit (BioVision). The cells were first transfected with clusterin siRNA solution as above. Then, the transfected cells were harvested and adjusted to a suitable cell suspension concentration with a 10% growth medium and then seeded into 24 well culture plates at a density of 2×10^4 cells/well. The cultures were incubated in a growth medium at 37 °C with a 5% CO_2 atmosphere. Then, withdrew a volume of 100 µL of culture medium at 24, 48, and 72 h of incubation to measure the LDH activity released from damaged cells in the culture supernatant. After that, transferred the culture medium to a flat-bottomed 96 well plate. A 100 µL of the mixture of lactate and tetrazolium salt was added to each well in triplicate. The plate was incubated at room temperature in the dark for 30 min. The absorbance reading of all the reactions was measured at 490 nm (reference wavelength at 620 nm) using a Sunrise™ ELISA microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The percentage (%) of cytotoxicity was calculated as described in the previous study [10].

Cell cycle analysis of clusterin-silenced colorectal cancer cells

Cell cycle analysis of the clusterin effect was performed by flow cytometry. As prepared above, a density of 2.0×10^5 clusterin-silenced cells/well in 6 well culture plates was incubated in triplicate at 37 °C with a 5% CO_2 atmosphere. Then, transferred the culture supernatants and the harvested cells from each well to new 15 mL falcon tubes after each incubation time-point (24, 48, and 72 h). The cell suspensions were centrifuged at 1,000 rpm for 5 min, then discarded the supernatant. The cell pellet was washed with phosphate-buffered saline (PBS) and pelleted the cell suspension again by centrifugation as above. Adjusted the number of cells in the suspension to 1×10^6 cells/ml. The suspension was then mixed gently with 500 µl of 70% cold absolute ethanol and stored at 4 °C to facilitate ethanol fixation later. The ethanol-fixed cells were centrifuged again at 4,500 rpm for 10 min. The cell pellet was rewashed as above and then resuspended in PBS. Finally, the suspension was centrifuged at 4,500 rpm for 10 min. The cells were then stained with 500 µl of FxCycle™ propidium iodide (PI)/RNase staining solution (Thermo Fisher Scientific) in the dark. The stained samples were then transferred to new sterile flow tubes and maintained on ice until the samples were analysed by FACSCantoTM II Flow Cytometry System (Becton Dickinson, NJ, United States).

Migration analysis of clusterin-silenced colorectal cancer cells

The scratch-wound cell migration assay of the clusterin effect in cells was conducted using the wound healing assay based on the wounded culture repopulation. The cells were first seeded at a density of 2×10^4 cells/well in 24 well culture plates. The cultures were then incubated at 37 °C with a 5% CO_2 atmosphere until the cells reached 80% confluence. The cultures were subjected to specific gene silencing for 72 h as above. A scratch (wound) on each culture maintained in the growth medium of silenced and non-silenced cells was made using a yellow pipette tip. Removed the cell debris, and a fresh medium in the culture was replaced. Each culture wound was photographed immediately after the scratch (0 h), 24, 48, and 72 h. Measure wound closure in each culture using ImageJ at each time-point by averaging three individual measurements. Each measurement was performed in triplicate and was repeated at least twice.

Gene expression analysis of clusterin-silenced colorectal cancer cells

Real-time PCR analysed the mRNA expression of cell proliferation- and migration-related genes in clusterin-silenced cells. A density of 2.0×10^5 silenced cells/well, as prepared above, were incubated in 6 well culture plates in triplicate at 37 °C with a 5% CO_2 atmosphere. Then, the culture supernatants and the harvested cells from each well were transferred to new 15 mL falcon tubes after each incubation time-point (24, 48, and 72 h). The cell suspensions were centrifuged at 1,000 rpm for 5 min, discarded the supernatant. The cell pellet was then subjected to extraction using TRIzol Total RNA Isolation Reagent (Thermo Fisher Scientific). The extracted total RNA concentration was then determined using NanoDrop® ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, MA, United States), whereby the high purity of the extracted RNA was stored at −80 °C until use. The extracted RNA integrity was checked using a 1% (w/v) agarose gel with an electrophoresis system and then was reverse transcribed to cDNA using a Tetro cDNA Synthesis Kit (Bioline). All primers used for the analysis, including Ki67, PCNA, TGFα, CCL5, EGF, BNIP3, and FGF4, were designed using Primer Express Software v3.0.1 (Thermo Fisher Scientific, MA, United States) (Table 1). All oligonucleotide primers (Integrated DNA Technologies) were packed in desalted lyophilised form. The primer stocks were dissolved in RNase-free water to generate a final 100 µM concentration of each primer solution. Stored both stock and working solutions of the primers at −20 °C until use. The expression of *Ki67*, *PCNA*, *TGFα*, *BNIP3*, and *FGF4* was also analysed because these genes correlated to cell proliferation, cell migration, and expression of *PPARα* and *PPARγ* [10]. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, MA, United States) in which prepared the PCR cocktail and performed the PCR reaction for the gene of interest (GOI) as described in the previous study [10]. The normalisation of the CT value (DNA amount) of GOI to the CT value of *β-actin* in a sample was performed. The expression level (fold change) was determined as the relation between the normalised GOI in the clusterin-silenced sample

Table 1: The list of primers used for mRNA expression analysis of cell proliferation- and migration-related genes by real-time PCR in this study.

Gene	Primer sequence
β-actin	Forward 5'-CATTGCCGACAGGATGCA-3'
	Reverse 5'-CCGATCCACACGGAGTACTTG-3'
Ki67	Forward 5'-AACTATCCTCGTCTGTCCCAACAC-3'
	Reverse 5'-CGGCCATTGGAAAGACAGAT-3'
PCNA	Forward 5'-AGAAGGTGTTGGAGGCACTCA-3'
	Reverse 5'-GGTTTACACCGCTGGAGGTAA-3'
TGFα	Forward 5'- CAGACCTTCCTACTTGGCCTGTAA-3'
	Reverse 5'- GACGGAGTTCTTGACAGAGTTTTG-3'
CCL5	Forward 5'-AGCCTCTCCCACAGGTACCAT-3'
	Reverse 5'-GGCAGTAGCAATGAGGATGACA-3'
EGF	Forward 5'-TGTGGTTCTCAGATTGGGCTATG-3'
	Reverse 5'-GATGAGGGCTTCAGCATGCT-3'
BNIP3	Forward 5'-AACTGGAGTCTGACTTGGTTCGTT-3'
	Reverse 5'-CCAGGATCTAACAGCTCTTCAGTGA-3'
FGF4	Forward 5'-CAACTACAACGCCTACGAGTCCTA-3'
	Reverse 5'-CCTTCTTGGTCTTCCCATTCTTG-3'

to non-clusterin-silenced sample (control) using the following formula: 2^(−ΔΔCt) [11].

Statistical analysis

All curves were generated using GraphPad Prism v8.2.1 for Windows (GraphPad Prism Software, Inc.). The statistical analysis was presented as the mean ± SD of triplicate determinations. One-way analysis of variance (ANOVA) was used for the data analysis with an additional Dunnett's multiple comparisons test. The confidence levels were indicated as statistically significant by p-value less than 0.05.

Results

Silencing clusterin mRNA in colorectal cancer cells

Optimisation of the transfection reagent in Caco2 revealed that 6 µl was the optimum volume to reduce the intensity of the clusterin protein band in the cells (Figure 1A). The *CLU* gene in Caco2 was used for optimisation because SW480 and SW620 expressed a lower level of clusterin than Caco2. As a result, clusterin-silenced Caco2 decreased the clusterin protein level from 1.08 to 0.81 units; only a faint protein band was detected (Figure 1B). Real-time PCR analysis revealed that the *CLU* gene at the mRNA level was also downregulated in Caco2 using the optimised gene silencing conditions for 72 h (Figure 1C). The reduction in clusterin mRNA expression was detected as a 0.8125-fold change ($p<0.05$) in clusterin-silenced Caco2, which was

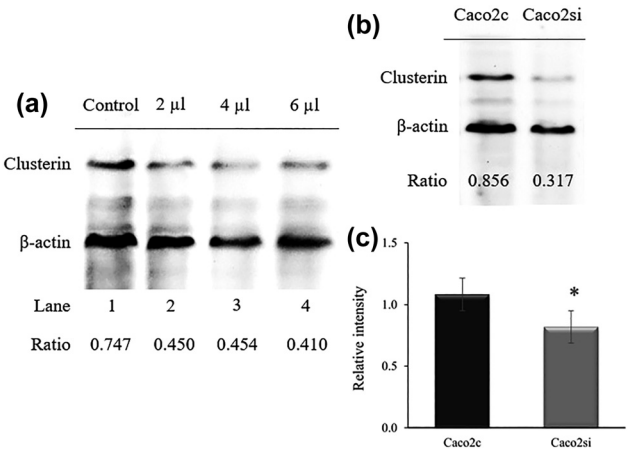


Figure 1: Silencing clusterin mRNA in Caco2.
(A) Optimisation of the transfection reagent for clusterin mRNA silencing, whereby 0 (control), 2, 4, and 6 µl of reagents were used for the optimisation. (B) The efficacy of clusterin mRNA silencing at the protein expression level in Caco2 by Western blotting. (C) The efficacy of *CLU gene silencing* at the mRNA expression level in Caco2 by real-time PCR. The data are expressed as the mean ± SD (n=3). Caco2c is indicated as Caco2 without undergoing gene silencing (control), whereas Caco2si is indicated as the cells had undergone clusterin mRNA silencing.

approximately 25%, compared to non-silenced Caco2 (control; 1.0625-fold change). The fold changes were detected less than 25% in clusterin-silenced SW480 and SW620 compared to the respective controls (data not shown).

Cytotoxic effect of clusterin-silenced colorectal cancer cells

The cytotoxic effect of clusterin-silenced SW480, SW620 and Caco2 revealed an increased % of cytotoxicity in the specific gene-silenced cells. The cytotoxic levels of clusterin-silenced SW480 at 24, 48, and 72 h were 19.8%, 26.6% ($p<0.01$) and 30.3% ($p<0.01$), respectively, compared to those of non-clusterin-silenced SW480 (control; Figure 2A). Next, the cytotoxic levels in clusterin-silenced SW620 at 24, 48, and 72 h were 14.7%, 12.2% ($p<0.05$) and 10.2% ($p<0.05$), respectively, compared to the respective control (Figure 2B). Unlike SW480 and SW620, the cytotoxic levels in clusterin-silenced Caco2 at 24, 48, and 72 h were 20.9% ($p<0.01$), 10.3% ($p<0.01$) and 3.7%, respectively, compared to the respective controls (Figure 2C). The cytotoxic level in clusterin-silenced Caco2 at 72 h was not statistically significant compared to the control because the specific gene silencing effect was not sustained up to 72 h.

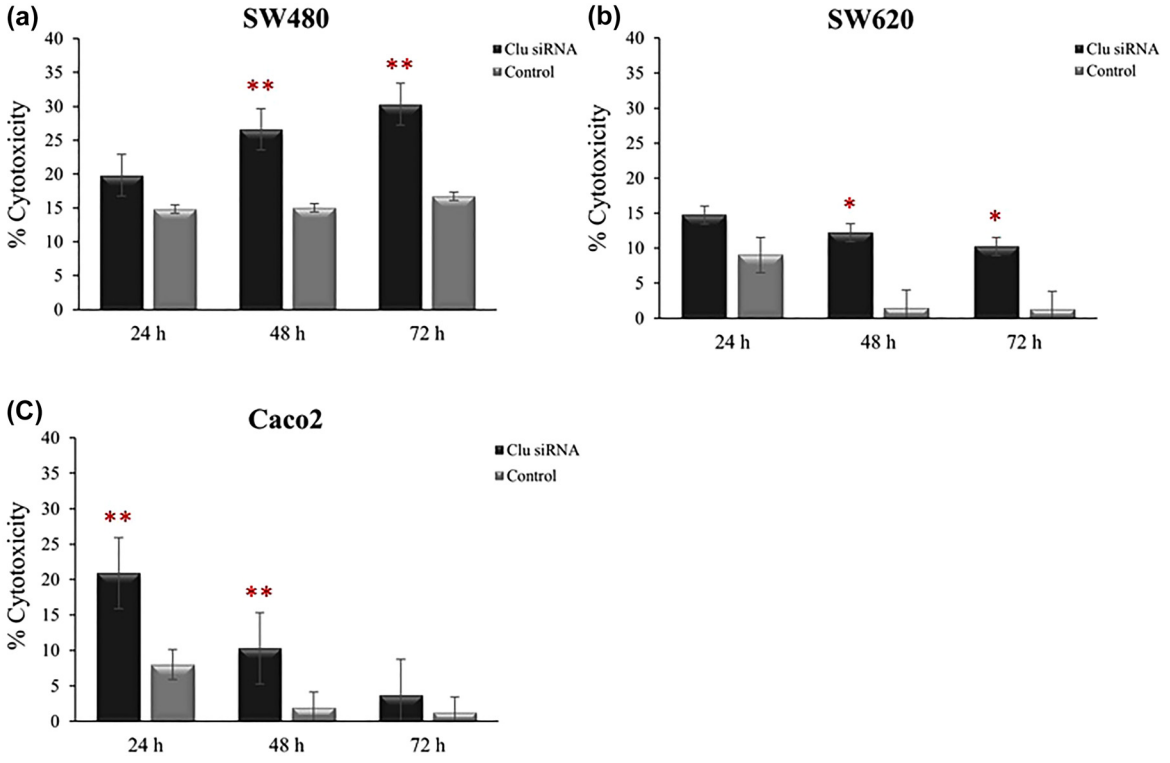


Figure 2: The percentage of cytotoxicity (%) in clusterin-silenced (a) SW480, (b) SW620, and (c) Caco2.
The cytotoxic level of clusterin-silenced colorectal cancer cells (Clu siRNA) compared to the cytotoxic level of non-clusterin-silenced colorectal cancer cells (control) at 24, 48, and 72 h. Three replications were carried out in at least two independent experiments. The data are represented as the mean ± SD; *p<0.05 and **p<0.01.

Cell cycle profiles in clusterin-silenced colorectal cancer cells

Cell cycle analysis, which detected the DNA content in clusterin-silenced cells, revealed that silencing the gene for 72 h did not induce obvious cell cycle arrest or potential apoptosis. Only 32.5% of non- (control) and 35.8% of clusterin-silenced SW480 were arrested in S phase (Figure 3A). Similarly, only 8.0%/56.1% of clusterin-silenced SW480 were arrested in G2/G1 phase, compared to 8.4%/59.0% of control at 72 h of incubation. The % of non- (control) and clusterin-silenced SW620 arrested in the S phase was 36.0%. In contrast, SW620 arrested in G2/G1 phases before (9.6%/54.3%) and after (10.6%/53.4%) the clusterin mRNA was silenced did not show a significant difference at 72 h of incubation (Figure 3B). Similar to SW480 and SW620, not much Caco2 was arrested in the S and G2 phases. The proportion of non- (control) and clusterin-silenced Caco2 arrested in the S phase was 54.0% and 50.7%, respectively (Figure 3C). In contrast, the proportion of control arrested in G2/G1 phases was 15.0%/30.9%. Thus, Caco2 arrest in G2/G1 phases remained constant at 14.5%/34.7% after silencing the specific gene in the cells for 72 h.

Cell migration rate in clusterin-silenced colorectal cancer cells

The migration rate of clusterin-silenced cells evaluated for 72 h revealed that incubation of clusterin-silenced SW480 for 72 h did not entirely cover the wound region (Figure 4A). The wound region covered as much as 32.3%, 52.7%, and 59.7% at 24, 48, and 72 h of incubation, respectively, in non-clusterin-silenced SW480 (control). However, only a tiny wound region appeared to cover in clusterin-silenced SW480 by 5.6%, 16.7%, and 23.7% at 24, 48, and 72 h of incubation, respectively. Comparison of the wound region in control (47.3%) to clusterin-silenced SW480 (83.3%) at 48 h of incubation revealed a statistically significant difference ($p<0.05$; Figure 4B). The assay also found that the wound region in control (40.3%) to clusterin-silenced SW480 (76.3%) at 72 h of incubation was significantly different ($p<0.05$). Similarly, incubation for 72 h did not entirely cover the wound regions of clusterin-silenced SW620 compared to non-clusterin-silenced SW620 (control). The wound region of the control covered as much as 22.9%, 41.4%, and 59.0% at 24, 48, and 72 h of incubation, respectively. In contrast, the wound

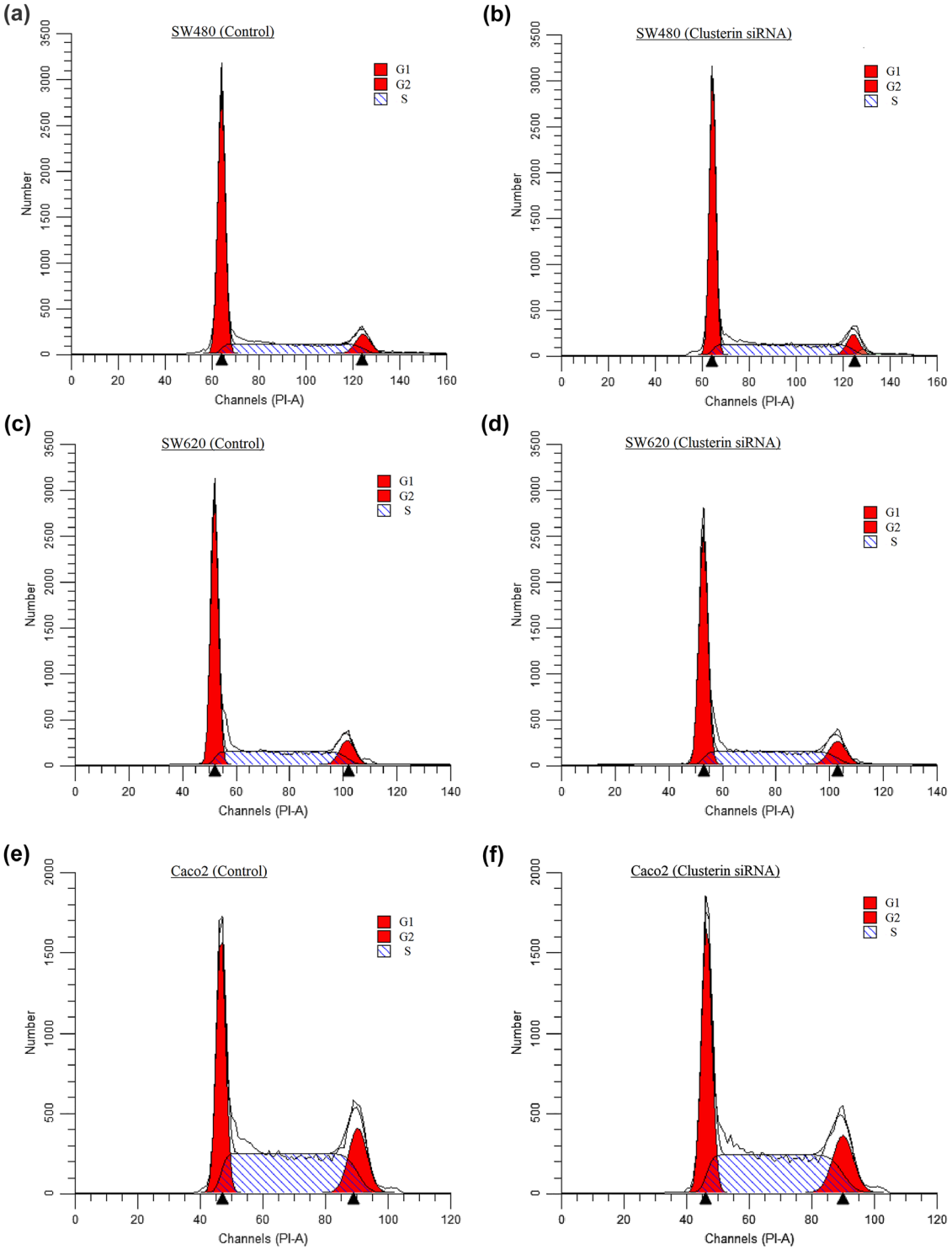


Figure 3: Cell cycle arrest in clusterin-silenced SW480, SW620, and Caco2 for 72 h. Non-silenced colorectal cancer cells were used as the control compared to clusterin-silenced cells (clusterin siRNA).
(A) SW480 control, (B) SW480 clusterin siRNA, (C) SW620 control, (D) SW620 clusterin siRNA, (E) Caco2 control, and (F) Caco2 clusterin siRNA. Three replications were carried out for each analysis in at least two independent experiments. Three phases of the cell cycle were detected: G1, G2 and S.

region was only covered by 7.9%, 10.3%, and 21.0% at 24, 48, and 72 h of incubation, respectively, in clusterin-silenced SW620 (Figure 5A). The wound region in control to clusterin-silenced SW620 at 48 h of incubation was observed as 58.6% and 89.7% ($p<0.01$), respectively (Figure 5B). In contrast, the wound region in control to clusterin-silenced SW620 at 72 h of incubation was 41.0% and 79.0% ($p<0.01$), respectively. Unlike SW480 and SW620, the wound region was observed to be covered more in Caco2 (control) at the end of the assay: 26.6%, 43.3%, and 70.2% at 24, 48, and 72 h of incubation, respectively (Figure 6A), indicating that incubation covered a higher wound region in non-clusterin-silenced Caco2. The wound region in clusterin-silenced Caco2 was observed to cover 6.6%, 20.7% and 42.1% at 24, 48, and 72 h of incubation. The wound region in control (56.7%) to clusterin-silenced

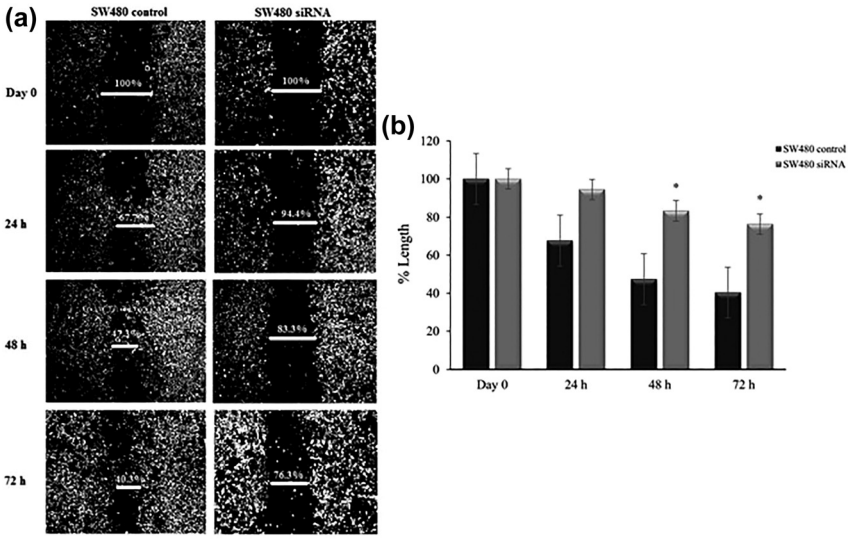


Figure 4: The migration of clusterin-silenced SW480.
(A) The migration rate in non-clusterin-silenced (control) and clusterin-silenced SW480 (siRNA) at 24, 48, and 72 h of incubation. Cell migration was estimated by measuring the wound region of the culture. (B) The graph of statistical analysis of non-clusterin-silenced (control) and clusterin-silenced SW480 (siRNA) at 24, 48, and 72 h of incubation. The data are expressed as the mean ± SD (n=3); *p<0.05.

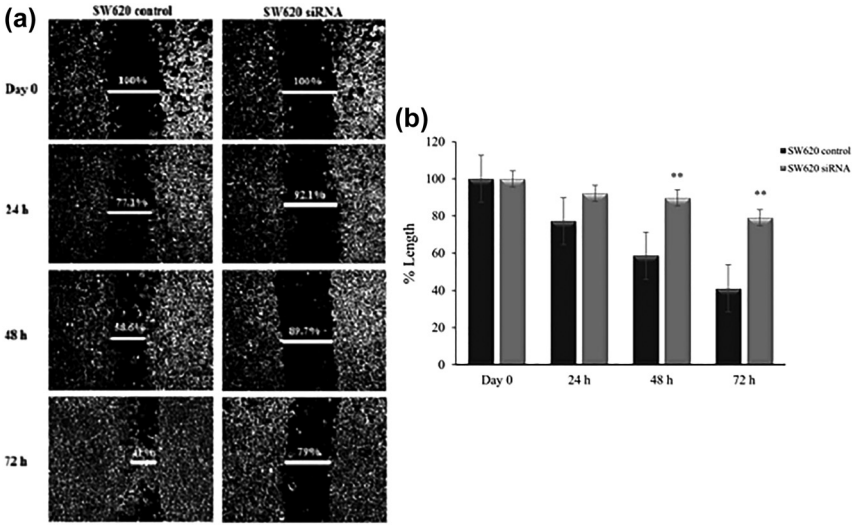


Figure 5: The migration of clusterin-silenced SW620.
(A) The migration rate in non-clusterin-silenced (control) and clusterin-silenced SW620 (siRNA) at 24, 48, and 72 h of incubation. Cell migration was estimated by measuring the wound region of the culture. (B) The graph of statistical analysis of non-clusterin-silenced (control) and clusterin-silenced SW480 (siRNA) at 24, 48, and 72 h of incubation. The data are expressed as the mean ± SD (n = 3); *p<0.05.

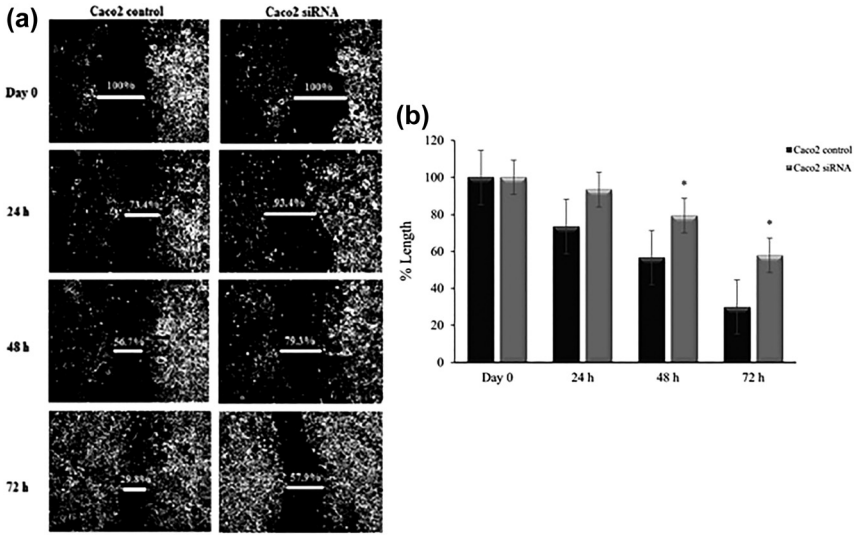


Figure 6: The migration of clusterin-silenced Caco2.
(A) The migration rate in non-clusterin-silenced (control) and clusterin-silenced Caco2 (siRNA) at 24, 48, and 72 h of incubation. Cell migration was estimated by measuring the wound region of the culture. (B) The graph of statistical analysis of non-clusterin-silenced (control) and clusterin-silenced SW480 (siRNA) at 24, 48, and 72 h of incubation. The data are expressed as the mean ± SD (n = 3); *p<0.05.

Caco2 (79.3%) at 48 h was observed to be a statistically significant difference (p<0.05; Figure 6B), whereas the comparison of the wound region in control to clusterin-silenced Caco2 at 72 h of incubation was revealed as 29.8% and 57.9% (p<0.05), respectively.

mRNA expression of genes in clusterin-silenced colorectal cancer cells

Evaluation of proliferation and migration markers in clusterin-silenced cells showed a decrease in the mRNA expression of the selected genes in SW480 and SW620 with clusterin silencing (Table 2). However, the reduction of most mRNA gene expression in clusterin-silenced cells, including Caco2, was not statistically significant compared with controls. The analysis observed that only FGF4 mRNA expression was downregulated to a 0.27-fold change in SW620, but it was not statistically significant. A similar phenomenon was observed for TGFα mRNA expression in clusterin-silenced Caco2, whereby the analysis found a reduction in TGFα (0.24-fold change) in the cells. The mRNA expression of CCL5, EGF and FGF4 was increased in clusterin-silenced Caco2. However, only the induction of CCL5 mRNA expression was statistically significant: a 2.3-fold change (p<0.05) compared to the control.

Table 2: Summary of the mRNA expression of genes related to cellular mechanisms in clusterin-silenced SW480, SW620 and Caco2 after 72 h. A 1.0-fold change indicated the relationship between the normalised GOI in clusterin-silenced and non-clusterin-silenced cells (control).

Gene	SW480	SW620	Caco2
Ki67	↓ 0.8-fold change	↓ 0.5-fold change	1.0-fold change
PCNA	1.0-fold change	↓ 0.6-fold change	↓ 0.5-fold change
TGFα	↓ 0.8-fold change	↓ 0.9-fold change	↓ 0.24-fold change
CCL5	↓ 0.5-fold change	↓ 0.4-fold change	↑ 2.3-fold change[a]
EGF	↓ 0.5-fold change	↓ 0.5-fold change	↑ 1.3-fold change
BNIP3	↓ 0.9-fold change	↓ 0.34-fold change	↓ 0.5-fold change
FGF4	↓ 0.38-fold change	↓ 0.27-fold change	↑ 1.3-fold change

Fold change > 1 is indicated as up-regulation, whereas fold change < 1 is indicated as down-regulation. The data are expressed as the mean ± SD (n=3). ↓ Down-regulation; ↑ up-regulation; [a]p<0.05.

Discussion

This study demonstrated that clusterin-silenced colorectal cancer cells increased the % of cytotoxicity significantly. However, the following analysis revealed that silencing the clusterin mRNA for 72 h did not induce cell cycle arrest or apoptosis in the cells. In addition, the wound-healing assay evaluated the inhibition of the cell migration rate after 72 h of incubation was likely due to the clusterin mRNA silencing. Next, the gene expression analysis revealed that CCL5 was induced in the clusterin-silenced Caco2-type colorectal cancer cells.

Clusterin was first linked to cell death in the rat ventral prostate after androgen deprivation [12]. Clusterin over-expression reaches a maximum at 3–4 days post-castration and coincides with the onset of massive cell death [12, 13].

Clusterin is a highly conserved glycoprotein with ubiquitous tissue distribution [1]. The clusterin level also rises in various malignant tumours compared with adjacent noncancerous tissue [14–17]. Clusterin over-expression has been closely associated with the development and progression of multiple cancer types [18]. The cancer types include gastric [17], ovarian [19], breast [20], bladder [21], colorectal [22], hepatic [23], laryngeal [24], and others [23]. Introducing the *CLU* gene into renal cell carcinoma or hepatoma cells enhances the metastatic cell potential and causes metastatic nodule enhanced formation in experimental animals [23].

Clusterin stimulates the migration and metastasis of cells by activating protein kinase B (Akt)-associated pathways in hepatocellular carcinoma [25, 26]. Many studies have verified that numerous proteins promote tumour cell biological functions by activating the AKT pathway via upregulation of secretory clusterin [1]. The AKT signalling pathway is believed to act downstream of secretory clusterin, e.g., the secretory clusterin-AKT signalling pathway is responsible for cisplatin resistance in human lung cancer [26]. Insulin-like growth factor-1 activates the phosphatidylinositol-3-kinase (P13K)/AKT signalling pathway via upregulation of secretory clusterin in lung carcinoma [27]. Knockdown of stathmin markedly inhibited secretory clusterin expression and the phosphorylation level of the AKT protein in cancer cells [28–32], confirming that the abnormal activation of the AKT signalling pathway was associated with stathmin and clusterin in cancers. Consistently, this study found that clusterin could be related to cell proliferation and migration.

SW480 was established from the primary adenocarcinoma of a male's colon. It is adherent cells express elevated levels of p53 protein, whereas the SW620 was derived from metastasis of the same tumour from which the SW480 was derived. SW480 and SW620 are cell lines, which can be considered subclones and have very similar drug responses. However, Caco2 is quite different from that expresses EGF. These differences could be due to genetic differences between the subclones and may provide important clues to the present study. In this study, the % of cytotoxicity in clusterin-silenced cells was the most potent in clusterin-silenced SW480, followed by specific gene-silenced SW620. The proliferation of Caco2 was the least influenced by clusterin mRNA silencing. This phenomenon indicates that clusterin may partially play a role in cell survival and death of Caco2 type of colorectal cancer cells. In laryngeal squamous cell carcinoma, the secreted form of clusterin is upregulated, and its expression is related to the invasiveness of these tumours [2]. The inhibitory role of *CLU* gene silencing in the invasive ability and growth of Hep2 human laryngeal squamous carcinoma cells significantly inhibited cell proliferation and promoted apoptosis. Furthermore, siRNA-mediated downregulation of clusterin mRNA expression decreases *in vitro* cell migration and invasion ability, similar to our present study on clusterin in colorectal cancer cells. Although no cell cycle arrest or apoptosis was observed, clusterin mRNA silencing indicates an essential role in protecting against carcinogenesis by eliminating genetically damaged cells [2]. Therefore, identification of the genes associated with clusterin in this mechanism is warranted.

Small interfering RNA-mediated clusterin mRNA silencing has been demonstrated to inhibit invasion and metastasis in breast [33, 34], lung [35, 36], and prostate [37] cancer cells. Similarly, our study found that clusterin mRNA silencing inhibited colorectal cancer cell migration. However, the study did not investigate the invasion property of clusterin mRNA silencing. The study found little difference in the clusterin mRNA silencing effect in SW480 and SW620, with the inhibition of the cell migration rate in clusterin-silenced SW620 was slightly more potent. This phenomenon explains Caco2 is a more aggressive colorectal cancer cell line, and the disease condition is unpredicted because the cells covered only a small cytotoxic effect and wound region after clusterin mRNA silencing. Perhaps the cellular mechanisms induced in all colorectal cancer cells are time dependent. Interestingly, the mRNA expression of CCL5 was observed to be significantly induced in clusterin-silenced Caco2. Further study on the correlation of this target gene in clusterin-silenced cells is warranted to aid the early detection of colorectal cancer patients infected with *S. mansoni* and, therefore, may provide a more efficient solution for disease treatment.

Silencing *CLU* gene has also been reported to be correlated with the expression of many other genes. For example, decreased clusterin mRNA and the corresponding protein levels in colorectal cancer cells are related to the over-expression of maternally expressed gene 3 (MEG3) [38]. Furthermore, clusterin over-expression rescues the compromised abilities of proliferation and metastasis induced by MEG3 over-expression, suggesting that MEG3 inhibits colorectal cancer progression by regulating clusterin activities [38]. The results also suggested that MEG3 functions as a tumour suppressor in colorectal cancer by regulating clusterin activities and underlying vitamin D anticancer activities [38]. *CCL5* and *CLU* are genes related to immune pathways, including antigen presentation, inflammation, apoptosis, and response to virus infection [39]. The CCL5 has been identified as a therapeutic target in colorectal cancer [40]. Our present study demonstrated that silencing clusterin induces the expression of *CCL5*.

Therefore, we believe that *CCL5* induction in clusterin-silenced cells has a role in maintaining cancer cell growth; both clusterin and CCL5 should be targeted for effective growth inhibition of Caco2-type colorectal cancer for our future drug design work. The expression of *Ki67*, *PCNA*, *TGFα*, *EGF*, *BNIP3*, and *FGF4* was identified as the genes correlated to the cell proliferation, the cell migration, and the expression of *PPARα* and *PPARγ* in IL-21-silenced colorectal cancer cells in our previous study [10]. However, the genes are likely, not correlated to silencing clusterin mRNA; the reduction or induction of these mRNA gene expressions was not statistically significant compared with controls in clusterin-silenced colorectal cancer cells.

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References

1. Shu F, Zou X, Tuo H, She S, Huang J, Ren H, et al. Stathmin gene silencing suppresses proliferation, migration and invasion of gastric cancer cells via AKT/sCLU and STAT3 signaling. Int J Oncol 2019;54:1086–98.
2. Wang Q, Cao W, Su Q, Liu Z, Zhang L. Clusterin silencing inhibits proliferation and reduces invasion in human laryngeal squamous carcinoma cells. World J Surg Oncol 2014;12:124.
3. Trougakos IP, Gonos ES. Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol 2002;34:1430–48.
4. Shannan B, Seifert M, Leskov K, Willis J, Boothman D, Tilgen W, et al. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. Cell Death Differ 2006;13: 12–9.
5. Chen XD, Halberg RB, Ehrhardt WM, Torrealba J, Dove WF. Clusterin as a biomarker in murine and human intestinal neoplasia. Proc Natl Acad Sci USA 2003;100:9530–5.
6. Andersen CL, Schepeler T, Thorsen K, Birkenkamp-Demtröder K, Mansilla F, Aaltonen LA, et al. Clusterin expression in normal mucosa and colorectal cancer. Mol Cell Proteomics 2007;6: 1039–48.
7. Abdalkareem EA, Tan GC, Abdalla HS, Lim BH, Khoo BY. Identification of specific proteins in colorectal cancer patients with Schistosoma mansoni infection as a possible biomarker for the treatment of this infection. Asian Pac J Trop Dis 2014;4: S720–24.
8. Imperlini E, Colavita I, Caterino M, Mirabelli P, Pagnozzi D, Vecchio LD, et al. The secretome signature of colon cancer cell lines. J Cell Biochem 2013;114:2577–87.
9. Ge P, Ong C, Abdalkareem A, Khoo BY, Yuan B. IFN-γ and IL-18 in conditioned media of parasite-infected host and IL-21-silenced colorectal cancer cells. Exp Ther Med 2021;21:103.
10. Abdalkareem EA, Ong CY, Lim BH, Khoo BY. Neutralizing FGF4 protein in conditioned medium of IL-21-silenced HCT116 cells restores the migratory activity of the colorectal cancer cells. Cytotechnology 2018;70:1363–74.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) method. Methods 2001;25:402–8.
12. Ammar H, Closset JL. Clusterin activates survival through the phosphatidylinositol 3-kinase/Akt pathway. J Biol Chem 2008; 283:12851–61.
13. Wang X, Liu R, Wang Y, Cai H, Zhang L. Effects of down-regulation of clusterin by small interference RNA on human acute myeloid leukemia cells. Int J Clin Exp Med 2015;8:20925–31.
14. Pucci S, Bonanno E, Sesti F, Mazzarelli P, Mauriello A, Ricci F, et al. Clusterin in stool: a new biomarker for colon cancer screening? Am J Gastroenterol 2009;104:2807–15.
15. Flanagan L, Whyte L, Chatterjee N, Tenniswood M. Effects of clusterin over-expression on metastatic progression and therapy in breast cancer. BMC Cancer 2010;10:107.
16. Rizzi F, Bettuzzi S. The clusterin paradigm in prostate and breast carcinogenesis. Endocr Relat Cancer 2010;17:R1–R17.
17. Bi J, Guo AL, Lai YR, Li B, Zhong JM, Wu HQ, et al. Overexpression of clusterin correlates with tumor progression, metastasis in gastric cancer: a study on tissue microarrays. Neoplasma 2010; 57:191–7.
18. Lee MH, Cho Y, Kim DH, Woo HJ, Yang JY, Kwon HJ, et al. Menadione induces G2/M arrest in gastric cancer cells by down-regulation of CDC25C and proteasome mediated degradation of CDK1 and cyclin B1. Am J Transl Res 2016;8:5246–55.
19. Yang GF, Li XM, Xie D. Overexpression of clusterin in ovarian cancer is correlated with impaired survival. Int J Gynecol Cancer 2009;19:1342–6.
20. Yom CK, Woo HY, Min SY, Kang SY, Kim HS. Clusterin overexpression and relapse-free survival in breast cancer. Anticancer Res 2009;29:3909–12.
21. Hazzaa SM, Elashry OM, Afifi IK. Clusterin as a diagnostic and prognostic marker for transitional cell carcinoma of the bladder. Pathol Oncol Res 2010;16:101–9.
22. Kevans D, Foley J, Tenniswood M, Sheahan K, Hyland J, O'Donoghue D, et al. High clusterin expression correlates with a

poor outcome in stage II colorectal cancers. Cancer Epidemiol Biomarkers Prev 2009;18:393–9.
23. Lau SH, Sham JS, Xie D, Tzang CH, Tang D, Ma N, et al. Clusterin plays an important role in hepatocellular carcinoma metastasis. Oncogene 2006;25:1242–50.
24. Redondo M, Esteban F, González-Moles MA, Delgado-Rodríguez M, Nevado M, Torres-Muñoz JE, et al. Expression of the antiapoptotic proteins clusterin and bcl-2 in laryngeal squamous cell carcinomas. Tumour Biol 2006;27:195–200.
25. Wang L, Cui Y, Ruge F, Jiang WG. Interleukin 21 and its receptor play a role in proliferation, migration and invasion of breast cancer cells. Cancer Genomics Proteomics 2015;12:211–22.
26. Zhang B, Zhang K, Liu Z, Hao F, Wang M, Li X, et al. Secreted clusterin gene silencing enhances chemosensitivity of A549 cells to cisplatin through AKT and ERK1/2 pathways *in vitro*. Cell Physiol Biochem 2014;33:1162–75.
27. Ma X, Bai Y. IGF-1 activates the P13K/AKT signaling pathway via upregulation of secretory clusterin. Mol Med Rep 2012;6:1433–7.
28. Wang ZQ, Cai Q, Hu L, He CY, Li JF, Quan ZW, et al. Long noncoding RNA UCA1 induced by SP1 promotes cell proliferation via recruiting EZH2 and activating AKT pathway in gastric cancer. Cell Death Dis 2017;8:e2839.
29. Pan T, Chen W, Yuan X, Shen J, Qin C, Wang L. miR-944 inhibits metastasis of gastric cancer by preventing the epithelial-mesenchymal transition via MACC1/Met/AKT signaling. FEBS Open Bio 2017;7:905–14.
30. Kim JW, Lee HS, Nam KH, Ahn S, Kim JW, Ahn SH, et al. PIK3CA mutations are associated with increased tumor aggressiveness and Akt activation in gastric cancer. Oncotarget 2017;8: 90948–58.
31. Wang C, Jiang J, Ji J, Cai Q, Chen X, Yu Y, et al. PKM2 promotes cell migration and inhibits autophagy by mediating PI3K/AKT activation and contributes to the malignant development of gastric cancer. Sci Rep 2017;7:2886.
32. Song Y, Li ZX, Liu X, Wang R, Li LW, Zhang Q. The Wnt/β-catenin and PI3K/Akt signaling pathways promote EMT in gastric cancer by epigenetic regulation via H3 lysine 27 acetylation. Tumour Biol 2017;39:1010428317712617.
33. Li J, Jia L, Zhao P, Jiang Y, Zhong S, Chen D. Stable knockdown of clusterin by vectorbased RNA interference in a human breast cancer cell line inhibits tumour cell invasion and metastasis. J Int Med Res 2012;40:545–55.
34. Niu Z, Li X, Hu B, Li R, Wang L, Wu L, et al. Small interfering RNA targeted to secretory clusterin blocks tumor growth, motility, and invasion in breast cancer. Acta Biochim Biophys Sin 2012;44: 991–8.
35. Chou TY, Chen WC, Lee AC, Hung SM, Shih NY, Chen MY. Clusterin silencing in human lung adenocarcinoma cells induces a mesenchymalto-epithelial transition through modulating the ERK/Slug pathway. Cell Signal 2009;21:704–11.
36. Yan Y, Luo K, Zhang H, Chai W. RNA interference-mediated secretory clusterin gene silencing inhibits proliferation and promotes apoptosis of human non-small cell lung cancer cells. Hepato-Gastroenterology 2013;60:70–5.
37. Bettuzzi S, Davalli P, Davoli S, Chayka O, Rizzi F, Belloni L, et al. Genetic inactivation of ApoJ/clusterin: effects on prostate tumourigenesis and metastatic spread. Oncogene 2009;28: 4344–52.
38. Zhu Y, Chen P, Gao Y, Ta N, Zhang Y, Cai J, et al. MEG3 activated by vitamin D inhibits colorectal cancer cells proliferation and migration via regulating clusterin. EBioMedicine 2018;30: 148–57.
39. Sharma A, Bhattacharya B, Puri RK, Maheshwari RK. Venezuelan equine encephalitis virus infection causes modulation of inflammatory and immune response genes in mouse brain. BMC Genom 2008;9:289.
40. Chang L-Y, Lin Y-C, Mahalingam J, Huang C-T, Chen T-W, Kang C-W, et al. Cancer Res 2012;72:1092–102.