RESEARCH ARTICLE

Targeting Cyclin E by Phellinus Linteus for Colon Cancer Therapeutics

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Abstract

Purpose: Colon cancer is a common malignant disease with a high rate of cancer-related deaths. Current conventional chemotherapies to treat advanced colon cancer are not effective and often have serious side effects. Thus, there is an urgent need for discovering new treatments with a high efficacy and less toxicity. Because phellinus linteus (PL) has been widely used in Asia to treat cancers, we in this study investigated how the polysaccharides (P-PL) extracted from PL effectively targeted colon cancer cells.

Methods: P-PL was used to test its effects on Human colon epithelial-like Caco-2 and cancerous HCT116 or HT29 cells IN VITRO and IN VIVO. The assays to analyze cell cycle progression, the expressions of G₁/S cyclins and function of mRNA binding protein HuR were conducted for investigating the molecular mechanisms of the anti-colon cancer action of P-PL.

Results: In this study, we demonstrated that P-PL treatment preferentially induced human colon cancer HCT116 or HT29 cells to apoptosis, but had a little cytotoxicity to the control, immortalized colon epithelial-like Caco-2 cells. P-PL-treated colon cancer cells accumulate in S phase of the cell cycle before underwent apoptosis. In this process, cyclin E (clnE) expression is decreased, which is due to the decrease of its mRNA stability. Furthermore, HuR function in treated cancer cells is perturbed.

Conclusion: Taken together, our findings strongly suggest that P-PL is a potential anti-cancer medicine that can block malignant growth of colon cancer and has less cytotoxic side effects.

Keywords: colon cancer, phellinus linteus, cell cycle, cyclin E, HuR, apoptosis.

Introduction

Colon cancer is the most common cancer, the advanced form of which has a very high rate of mortality [1-3]. The etiology of colon cancer includes a broad collection of genetic, dietary and environmental factors [4, 5]. Through multiple steps of genetic or epigenetic alterations that perturb the balance controlling cell growth and apoptosis, colorectal cancer cells are evolved. The cancerous cells undergo hyperplasia, onset of adenomas and in situ carcinomas, and then progress to invasive tumors. During colon tumorigenesis, oncogenes (such as B-Raf or K-Ras) are often activated and, tumor suppressors are either lost or mutated [6, 7]. The activation of oncogenic signalling pathways augments the expression of growth-related factors to promote growth or angiogenesis. Concomitantly, another major cause of colon cancer etiology is the perturbation of pathways controlling cell cycle checkpoints or apoptosis [8]. Together, these factors contribute to colon adenomas to evade apoptosis and transform into malignant forms of the tumor.

Phellinus Linteus (PL) is a medicinal fungus and belongs to the Hymenochaetaceae Basidiomycetes that mainly grow in Asia [9-14]. The extract from PL has been widely used in Asia to prevent or to treat malignant diseases, which is recently recognized by the Western for cancer therapy [15-17]. PL extract consist of a mixture of various bioactive substances that have the complicated chemical natures of botanical constituents. Through the extraction and purification, the main active substances of PL have been revealed to be the polysaccharides at the molecular weight range of 150 kD-170 kD [3, 10], [14-16]. The polysaccharides of PL (P-PL) possess the immune-stimulating and immune-modulatory functions, by stimulating B lymphocytes and boosting body immune system against malignant diseases [15, 16]. P-PL treatment could dramatically improve the antigen presentation process for lymphocytes, resulted in increasing the expression of cell surface markers (for example, MHC I/II) of dendritic cells with rapid migration into lymphoid tissues [15, 16]. Recently, in vivo and in vitro studies (including ours) demonstrated that P-PL possessed a direct anti-cancer activity against different types of tumors, including colon cancer [17, 19]. P-PL-mediated cytotoxicity, caspase cascade was activated [17]. Although these studies suggested that P-PL is a promising candidate for cancer prevention or treatment, the underlying mechanisms by which P-PL directly blocks cancer initiation and progression remain not fully understood.
In cancer, RNA binding proteins (some of which transnationally regulate growth factors/proto-oncogenes/cytokines) are overexpressed or hyperactive [20-22]. The human version of the RNA-binding protein HuR belongs to ELAV family (the embryonic lethal abnormal vision), and is discovered as an antigen in patients with paraneoplastic disorders [23, 24]. HuR contains 3 RNA recognition motifs (RRM) with the high binding ability of AU-rich element (ARE) in the 3’UTR of mRNAs. Upon binding to ARE sequence, HuR stabilizes genes or mRNAs [22, 25-27]. This RNA-binding protein is primarily located in the nucleus and traffics to the cytosol to stabilize mRNAs there [22, 25-27]. The growth promotion mediated by overexpression of HuR was shown to link to its cytosolic translocation, where HuR associated with targeted ARE-containing mRNAs of growth- or cell cycle-related genes and stabilized them [23, 28-30]. Many mitogenic signal transducers that activate major intracellular pathways, such as ERK/MAPK, PI3K/Akt or Wnt signaling pathways were shown to sustain cellular activities, via promoting the cytoplasmic translocation of HuR and further the stability of the ARE-containing mRNAs of growth-related factors [18, 31, 32]. Because HuR regulates genes that promote cell growth, cell cycle progression (in particular, cyclins) and metastasis, the suppression of this mRNA stability regulator are considered as an attractive strategy for cancer treatment.

Cellular respond to geno-stress or cellular insults by triggering cell cycle arrest, damage repair or death is frequently perturbed during tumorigenesis, resulted in losing cell cycle restrictions, genetic instability and defects in the induction of apoptosis [24]. Cyclin E (clnE) is a cell cycle factor and mainly regulates S phase progression of the cell cycle [22]. ClnE mRNA contains the ARE sequence at its 3’UTR, and therefore, is subjected to be regulated by HuR [15, 33]. Dereglulation of clnE is often associated with cancer initiation and development. In breast cancer, it was shown that clnE was overexpressed, due to the increase of its stability, which is mediated in part by HuR [34].

In the study, we investigated how P-PL treatment, through perturbing HuR function, destabilized clnE and further induced S phase accumulation for the induction of apoptosis in colon cancer cells. We analyzed clnE protein expression, its mRNA stability, HuR abundance and cellular localization in colon immortalized Caco and cancerous cells. We also tested the influence of P-PL treatment on cell cycle progression, focusing on S phase of the cell cycle. We found that the level of clnE expression in colon cancer cells was rapidly decreased in response to P-PL treatment, which was due to the destabilization of its mRNA. In this process, HuR cytosolic translocation from the nucleus in treated cancer cells was partially mitigated, which might cause its insufficiency to binding to and stabilize clnE mRNA. As the results, colon cancer cells, but not the control cells, accumulated in S phase of the cell cycle, leading to the induction of apoptosis. Our study implicates that P-PL appears a promising medicine for treating colon cancer, perhaps for other types of cancer.

Materials and Methods

Cell Lines and Reagents

The human immortalized Caco-2 and colon cancer HCT116 or HT29 cells were purchased from American Tissue Culture Collection (Rockville, MD). All the cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL of penicillin, 100 mg/mL of streptomycin. P-PL was extracted and purified by Dr. S-H Kim (Kyung Hee University, South Korea). Briefly, Phellinus linteus bodies (Kyudong, Seoul, Korea) at about ~30g were pulverized, immersed, extracted with hot water (600 ml) at 95°C for 2 h and filtered using Whatman filter paper (GE Healthcare Bio-Sciences Corp.). Water-soluble polysaccharides were concentrated to approximately 1/10 of the original volume with a rotary vacuum evaporator at 55°C. The concentrated extract was mixed with 96% ethanol, stirred vigorously, and left overnight at 4°C. The precipitated P-PL was centrifuged at 11300 × g, and the supernatant was discarded. The remnant fraction was lyophilized to get approximately 60 mg of P-PL.

DNA Fragmentation and Cell Cycle Assays

Flow cytometric analysis was performed with a FACScan machine (Becton Dickenson, Mountain View, CA). The data analysis and display were performed with the Cell-Fit software program (Becton Dickenson). Cell-Fit provides data from the flow cytometer and real-time statistical analysis of the data. After various treatments, cells (1 x 10^6/ml) were washed with 1 x PBS, fixed with 70% ethanol. Subsequently, cells were stained with propidium iodide (0.1 ug/ml) containing RNase at 1.5 ng/ml. The stained samples were kept at 4°C overnight before flow cytometric analysis for either measuring percentages of less G1, DNA contents of apoptotic cells or DNA profiles of cells in different phases of the cell cycle.

Double Thymidine Block

Cells were cultured in the growth medium to 50%-60% confluence. The thymidine blocking solution (Sigma, St. Louis, MO) was then added in the cultures. After 19 h of culturing, the media of the cultures were removed. After grown in the fresh growth media for 9 h, the thymidine solution was added in the cultures that were grown for another 16 h when majority of cells were blocked in the boundary of S phase of the cell cycle.

Immunoblotting Analysis

After treatments, cells were washed in 1 x PBS, lysed in the lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-X114, 0.5% sodium deoxycholate, 0.1% SDS, containing 1 mM phenylmethylsulfonyl fluoride, 1 ng/ml of aprotinin, 1 ng/ml of leupeptin, 1 mg/ml of pepstatin A) and left on ice for 30 min. The total protein concentrations in cell lysate were normalized. Lysis samples with equal amount of total proteins were separated on a 10% SDS–PAGE gel and subsequently transferred to a nitrocellulose membrane.
All the antibodies used were purchased from Cell Signaling Tec. (Beverly, MA).

**Quantitative Real Time-PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the instruction provided by the manufacturers. cDNA was prepared using 1 μg of total RNA extracted (iScript cDNA synthesis kit, Bio-Rad), and then subjected for the amplification by quantitative real time-PCR using Applied Bio system Step One Plus in the presence of SYBR Green JumpStart (Sigma-Aldrich). Ribosomal 18 S RNA was used as the normalization control. The n-fold change in mRNA expression was then determined. The human clnE sense primer is: 5’-gtctctgctgaatgtatacatgc-3’ and the antisense primer is: 5’-cctatttgttcagacaacatggc-3’.

**Polyadenosine Tail Analysis**

After blocking DNA synthesis by ATC, total RNAs at different time points of the block were isolated. After reverse transcription was done using oligo (dT) and an oligo (dT), cDNAs were used for PCR with the clnE 3’UTR specific primer: 5’-gatgctgctatggaaggtgc-3’. The resulted PCR products were resolved on agarose gels and visualized by ethidium bromide staining.

**Rip Rna-Binding Protein Immunoprecipitation**

After the treatments, the cytosolic fractions were isolated from the cells and then analyzed using Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Burlington, MA)

**Statistical analysis**

Statistical analysis was performed using a two-tailed Student’s t test for comparison of two groups or a one-way analysis of variance for comparison of more than two groups followed by Tukey’s multiple comparison tests. Tumor-free probabilities were estimated using Kaplan-Meier Method and were compared among groups. Standard deviations are displayed in the figures. A p value <0.05 was considered significant.

**Results**

**High Doses of P-PL Sensitized Human Colon Cancer Cells to Apoptosis**

Figure 1(a): a Caco-2, HT29 and HCT116 cells were treated with different concentrations of P-PL for 48 h, and DNA fragmentation assay was then performed. Error bars represent standard deviation (SD) from 5 independent experiments (p<0.05)  

Figure 1(b): HCT116 cells were inoculated subcutaneously into the nude mice. Four days later, P-PL was injected intraperitoneally every 4 days. One week after the injection, the diameters of the tumors were measured weekly for consecutive 4 weeks and plotted. The error bars represent the SD (p <0.05) (left panel).
P-PL was demonstrated to possess cytotoxicity against human cancers [10, 12]. However, the underlying mechanisms by which P-PL antagonize tumors has not been fully explored. To explore P-PL therapeutic natures and mechanisms, human immortal colon Caco-2 and malignant colon HTCL116 or HT29 cells were employed. The cells were treated with different concentrations of P-PL for 48 h, and DNA fragmentation assay was then conducted (Fig. 1a). The treatment of P-PL at any testing doses did not induce apoptosis in control Caco-2 cells. In comparison, HCT116 or HT29 cells started to be susceptible to apoptosis at 50 μg/ml of P-PL. The susceptibility of the cancer cells to apoptosis was dramatically enhanced with increases of P-PL doses (> 50 μg/ml). More than 30% of the cancer cells underwent apoptosis after being treated with 150 μg/ml of P-PL, the dose of which was used in following experiments. The same results were obtained from Annexin V analysis (data not shown). The data suggested that P-PL at high doses was cytotoxic specifically to colon cancer cells.

The effect of P-PL on tumor formation was then analyzed using xenograft assay. HCT116 cells were inoculated into the nude mice (5 mice/group) subcutaneously. Four days later, P-PL (60 mg/kg) was injected intraperitoneally every 4 days [33]. One week later when the signs of the tumor formation were detected, the diameters of the tumors were measured weekly for consecutive 4 weeks (Figure 1b). Overall, the growth rate of xenografted tumors in the mice received P-PL injection was much slower than that in untreated mice.

Colon Cancer Cells Persistently Accumulated In S Phase and Express A Decreased Level of ClnE in Response to PLGL Treatment

During conducting DNA fragmentation assay using a flow cytometer, we noticed that an increasing population of P-PL-treated colon cancer cells, but not treated Caco-2 cells, was resided in S phase of the cell cycle before apoptosis occurred. In order to determine the significance of S phase accumulation, Caco-2, HT29 and HCT116 cells were synchronized at the beginning of S phase by the double thymidine block. At the end of the first exposure to thymidine, the cells were treated with P-PL (150 μg/ml), followed by the second thymidine block. The cells were harvested at different time points following being released from the synchronization and subjected to flow cytometer analysis. One hour after the releasing, the majority (> 80%) of untreated or P-PL-treated cells was detected in S phase, as expected (Figure 2a). The percentages of untreated Caco-2, HT29 or HCT116 cells in the S phase were rapidly reduced afterwards. The kinetics of P-PL treated Caco-2 cells exiting from S phase was similar as that of untreated cells. Notably, the kinetics of P-PL treated HT29 and HCT116 cells exiting from S phase were slower, as more than 40% of the cells still did not enter to the next phases of the cell cycle 8 h after the releasing from the block.

ClnE is both required and rate limiting for the entry into and exit from S phase. The expression of clnE is periodic, which peaks at G1/S transition and decayed at the end of S phase [35, 33]. Therefore, we analyzed clnE expression patterns in untreated and asynchronous cells as well as P-PL treated cells released from thymidine block at different time points, using immunoblotting (Figure. 2b). The expected clnE expression pattern was detected in Caco-2 cells, which was peaked 1 h after the releasing from thymidine block and returned to a comparable level as that of the asynchronous control 8 h later. In P-PL-treated colon cancer HT29 and HCT116 cells, 1 h after the release from thymidine block, clnE expression level was increased (> 2 folds), which was similar as the control Caco-2 cells. However, 4 h after the releasing, the expression levels of clnE in P-PL-treated colon cancer cells were significantly reduced and almost undetectable.

Subsequently, the stability of clnE in the cells was tested (Figure. 2c). The kinetics of ClnE degradation in untreated or P-PL-treated Caco-2 cells were similar, as the expression level started to decrease 6 h after the block of protein synthesis by cyclo heximide (CHX). The kinetics of ClnE degradation of untreated colon cancer cells was comparable with that seen in Caco-2 cells. However, clnE in HCT116 or HT29 cells became very unstable in response to P-PL treatment, large amounts of which were disappeared 4 h after the block of protein synthesis and became almost undetectable 6 h later.

Next, we analyzed the expressions of other G1/S cyclins D and A. The expression levels of cln D (Figure. 2d, left panels) or cln A (Figure. 2d, right panels) were similar in untreated Caco-2 and colon cancer cells as well as in the cells treated with P-PL. The kinetics of the degradations of these cell cycle regulators were also examined by immunoblotting. After P-PL treatment, the degradation patterns of cln D (Figure. 2e) and A (Figure. 2e) in P-PL-treated HCT116 cells were similar as that of untreated cells. The degradations of clns D and A in untreated or P-PL-treated HT29 cells were also examined and the similar results were obtained (data not shown).

ClnE Mrna Became Unstable In P-PL-Treated Colon Cancer Cells

In order to determine at which level clnE stability was perturbed, the kinetics of clnE at the transcription level was analyzed, using quantitative real time PCR (Figure. 3a). After the addition of actinomycin D (ATC) to block the gene transcription machinery, the level of clnE in P-PL-treated HCT116 or HT29 cells was rapidly reduced, and almost reached a baseline level at 6 h of ATC blocking. In comparison, the amounts of clnE transcripts in P-PL-treated Caco-2 or untreated cells were relatively stable and a moderate amount of clnE could still be detected 6 h after the block of the transcription process. The degradation of cln D or A in Caco-2, HCT116 and HT29 cells was also analyzed. The expressions of these genes in P-PL-treated colon cancer cells were relatively stable (data not shown). The data suggested that the decrease of clnE stability in P-PL-treated colon cancer cells was mainly affected at the transcription or post-transcription level.

Adenylation is an important step for most of mRNAs to be stabilized and further translated [22, 25-27]. Because clnE was
unstable in P-PL-treated colon cancer cells, it led us to test if
clnE destabilization occurred at the post-transcription level. The
lengths of clnE mRNA polyadenosine A tail (poly A) in untreated
and P-PL-treated HCT116 cells were analyzed by ligase-mediated
poly A tail assay with a specific primer selected from cln E 3'UTR
(Figure. 3b). Two hours after the block of DNA synthesis by ATC,
the lengths of clnE mRNA poly A tail in untreated HCTC116 cells
were similar as that at 0 h. In comparison, clnE mRNA poly a
tail in PLGL-treated HTC116 cells was barely detectable at any
testing time points after ATC block. The data suggests that the
adenylation of clnE mRNA in colon cancer cells was mitigated by
P-PL treatment, which suggested that the destabilization of clnE
happened at the level of regulating mRNAs.

Ability of HuR to bind to the CLNE 3’UTR is mitigated in
P-PL-treated colon cancer cells

AREs at the 3’UTR regulate adenylation, translation and
degradation of mRNAs. HuR is a mRNA-binding protein and
resides in the nucleus. In order to function, HuR translocate from
the nucleus to the cytosol where it binds to mRNAs containing
the ARE sequence at their 3'UTR and stabilizes them [22, 25-
27]. ClnE mRNA was shown to be one of HuR targets [35].
We compared the levels of HuR expression in P-PL-treated
Caco-2 and HCT116 cells after they were released from the
thymidine block, by immunoblotting. (Figure. 4a). Similar
amounts of HuR were detected in total cell lysates from both
treated cells at any time points of CHX exposure were analyzed by immunoblotting. Actin is a loading control.
A high and comparable level of HuR was seen in P-PL-treated Caco-2 cells at 0, 2 or 4 h of thymidine releasing, and only a low amount of this mRNA binding protein was detected in treated HCT116 cells (Figure 4a, middle two panels). On the contrary, nuclear HuR level in P-PL-treated HCT116 cells remained high (Figure 4a, bottom two panels).

Next, we examined the binding level of cytosolic HuR in P-PL-treated cells to clnE mRNA. Magna RIP RNA-binding protein immunoprecipitation kit was used (Figure 4b). Starved HCT116 cells that were re-fed with the growth medium containing 10% of newborn calf serum were served as the positive control. After the treatments, the cytosolic fractions were isolated from the cells for the analysis. HuR association with clnE mRNA was barely detected in the cytosolic fraction of untreated Caco-2 or HCT116 cells. After P-PL treatment, the immunoprecipitates pulled out by anti-HuR Ab from the cytosolic fraction of P-PL-treated Caco-2 cells or positive control HCT116 cells were contained clnE mRNA that could be amplified by reverse transcription PCR. However, the interaction of HuR and 3'UTR of clnE mRNA was hardly seen in the cytosolic fraction of HCT116 cells treated with P-PL, further indicating that HuR function was perturbed in treated colon cancer cells.

To further determine whether HuR cytosolic translocation in the colon cancer cells was mitigated in our experimental setting, the subcellular localization of HuR in the cells with or without P-PL treatment was analyzed by immunoblotting (Figure 4c). HuR was absent in the cytosolic fractions of untreated Caco-2 and HCT116 cells, but present in the nuclear fractions. In response to P-PL treatment, HuR was translocated to the cytosol in Caco-2 cells as well as of the positive control of HCT116 cells. In comparison, only a small amount of HuR was detected in the cytosol of P-PL-treated HCT116 cells. To further confirm this, the subcellular localization of HuR in our experimental setting was analyzed by immunohistochemistry (Figure 4d). Consistently, HuR was mainly detected by the antibody in the nucleus of untreated Caco-2 and HCT116 cells. After P-PL treatment, HuR in Caco-2 cells was translocated to the cytosol, which did not occur in HCT116 cells. The data suggested that P-PL treatment at least in part sequestered HuR in the nucleus of colon cancer cells, which appeared contribute to destabilize clnE mRNA.

**Discussion**

Colon cancer is one of the most common cancers with a high rate of cancer mortality worldwide [36, 2, 3]. Because of the dismal prognosis of advanced colon cancer and severe side effects of current existing conventional chemo-therapy, there is an urgent need for developing efficient treatments with...
less toxicity. For this purpose, we initiated the investigation of the effect of P-PL polysaccharides on colon cancer. We demonstrate a link among rapid decay of clnE, aberrant S phase accumulation and onset of apoptotic crisis in colon cancer HCT116 and HT29 cells following P-PL treatment. Briefly, compared with colon immortalized Caco-2 cells, the level of clnE expression in the colon cancer cells is decreased in response to P-PL treatment, because its mRNA stability is weakened. Furthermore, the function of the mRNA-binding protein HuR is perturbed in P-PL-treated colon cancer cells, reflected by its inadequate translocation from the nucleus to the cytosol. The RNA-binding protein immunoprecipitation assay reveals the lack of the binding of HuR to the ARE sequence of the 3′UTR of clnE mRNA in the cytosol of P-PL-treated colon cancer. Taken together, the results suggest that P-PL treatment interferes with HuR function, which may contribute to destabilizing clnE mRNA and further to S phase accumulation. As the result, apoptosis is preferentially induced in P-PL treated HCT116 and HT29 colon cancer cells. Our findings strongly suggest that this Asian medicinal extract has its potential for treating colon cancer patients with less damaging side effects.

Like other traditional Asian medicines, the use of P-PL to treat human malignancies is often based on empirical practice with lacking experimental research to support its clinical implement. P-PL was considered to be non-toxic in general, and block malignant cell growth through affecting cell cycle checkpoints [14]. In murine lung epithelial cells, P-PL treatment triggered G1 checkpoint by blocking the expression of cyclin D1 as well as activation of cell cycle dependent kinases 4 and 6 [17]. Because G1 cell cycle restriction in lung tumor cells was

![Image](image-url)
impaired, it allowed P-PL treatment specifically activating cell death program. It was also reported that the treatments of P-PL and doxorubicin acted in synergy to elicit apoptosis in prostate cancer cells and block the cancer cells to form colonies in soft agar medium [17]. In current study, we demonstrated that the high dose of P-PL was cytotoxic to colon cancer cells, but had a minimal killing effect on immortalized Caco-2 cells. In this apoptotic process, S phase regulator clnE was targeted by P-PL, which led to the occurrence of S phase crisis and further elimination of colon cancer cells.

Cyclin E is upstream of the retinoblastoma (Rb). During cell cycle progression, Rb is phosphorylated by cyclin D-Cdk4/6 during late stages of G phase and by cyclin E-Cdk2 in early stages of S phase [37, 38]. Afterwards, E2F is released from being sequestered by Rb to promote S phase progression [37, 38]. E2F is a transcription factor that regulates the expression of genes involved in the regulation of S phase of the cell cycle. Because clnE promoter contains E2F binding sites, it is one of E2F target genes, [39, 40]. By phosphorylating to inactivate Rb, clnE reinforces its own expression through a positive feedback loop. Upon the inactivation of Rb and subsequent activation E2F, cln E expression and activity are augmented, which promote cells resided in S phase to move to the next phase of the cell cycle. It is conceivable that P-PL treatment targets clnE degradation machinery and induces a rapid decay of this cyclin, which causes colon cancer cells to aberrantly accumulate in S phase to trigger apoptosis. ClnE, together with E2F, are essential mediators of S phase of the cell cycle in Drosophila [41, 42]. Thus, the role of E2F in P-PL-mediated accumulation of colon cancer cells in S phase should be in concert with aberrant clnE expression and indispensable.

HuR is a ubiquitously expressed mRNA binding protein and its activity is closely regulated by its subcellular localizations. This mRNA regulator is frequently overexpressed or hyperactive in various types of cancers [43, 18, 32]. Studies show that HuR is primarily located in the nucleus and traffics to the cytosol in response to genotoxic or oncogenic stress among other types of stimulation [30, 18, 32]. An active HuR Tran locates to the cytosol where it binds to targeted ARE-containing mRNAs and stabilizes them. The activation of mito genic pathways, such as Raf/ERK1/2, PI3K/Akt or Wnt signaling, has been shown to be able to stabilize ARE-containing mRNAs of growth-related genes by the active and cytosolic HuR, and further up regulate the encoded protein expressions [30, 18, 32]. In our current investigation, we demonstrated that P-PL treatment promoted the nuclear HuR to shuttle to the cytosol of control Caco-2 cells, the process of which was partially blocked in colon cancer cells. Because HuR is crucial for the regulation of genes and their protein products, and further for related cellular activities, the mitigation of HuR cytosolic translocation in P-PL-treated colon cancer cells would certainly affect its function in stabilizing mRNAs of cell cycle regulators, such as clnE in our experimental setting.

The stability of mRNAs is an important element in the regulation of the expression of gene and encoded proteins, which would have crucial impacts on subsequent cellular activities. The mRNA binding site of ARE is the cis promoting element that regulates the stability of many genes [33]. Our study showed that HuR in starved colon cancer cells re-fed with the growth medium was functional. Therefore, HuR in the colon cancer cells, in particular HCT116 cells, appeared functional. It appears that P-PL treatment activates a negative regulatory machinery that not only interferes with its binding to clnE mRNA, but also partially prevents its cytosolic translocation in colon cancer cells. Taken together, the data suggest that clnE and HuR are the key targets of P-PL for killing the colon cancer cells. We are in the process to identify inhibitory factors that may be activated by P-PL treatment in the nucleus to block HuR nuclear export as well as in the cytosol to compete with its binding to the mRNA in colon cancer cell.

Conclusion

The current study provides the evidence that the decrease of mRNA stability is specifically linked to the rapid degradation of clnE in P-PL-treated colon cancer cells. The study also demonstrates that P-PL, via perturbing HuR function, weakens clnE mRNA stability, which contributes to its anti-colon cancer activity. Overall, our study highlights the potential significance of P-PL for treating colon cancer patients, which may have less side effects.

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