

ORIGINAL ARTICLE

Pre-45s rRNA promotes colon cancer and is associated with poor survival of CRC patients

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One characteristic of cancer cells is the abnormally high rate of cell metabolism to sustain their enhanced proliferation. However, the behind mechanism of this phenomenon is still elusive. Here we find that enhanced precursor 45s ribosomal RNA (pre-45s rRNA) is one of the core mechanisms in promoting the pathogenesis of colorectal cancer (CRC). *Pre-45s rRNA* expression is significantly higher in primary CRC tumor tissues samples and cancer cell lines compared with the non-tumorous colon tissues, and is associated with tumor sizes. Knockdown of *pre-45s rRNA* inhibits G1/S cell-cycle transition by stabilizing p53 through inducing murine double minute 2 (MDM2) and ribosomal protein L11 (Rpl11) interaction. In addition, we revealed that high rate of cancer cell metabolism triggers the passive release of calcium ion from endoplasmic reticulum to the cytoplasm. The elevated calcium ion in the cytoplasm activates the signaling cascade of calcium/calmodulin-dependent protein kinase II, ribosomal S6 kinase (S6K) and ribosomal S6K (CaMKII-S6K-UBF). The activated UBF promotes the transcription of *rDNA*, which therefore increases *pre-45s rRNA*. Disruption of CaMKII-S6K-UBF axis by either *RNAi* or pharmaceutical approaches leads to reduction of *pre-45s rRNA* expression, which subsequently suppresses cell proliferation in colon cancer cells by causing cell-cycle arrest. Knockdown of APC activates CaMKII-S6K-UBF cascade and thus enhances *pre-45s rRNA* expression. Moreover, the high expression level of *pre-45s rRNA* is associated with poor survival of CRC patients in two independent cohorts. Our study identifies a novel mechanism in CRC pathogenesis mediated by *pre-45s rRNA* and a prognostic factor of *pre-45s rRNA* in CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide.¹ One million new CRC cases are reported annually, and the disease-specific mortality rate is nearly 33%.² With the changes of living and dietary habits, the incidence of CRC is rapidly increasing in Asian in the past decade. However, the molecular mechanisms that drive the development of CRC remain elusive.

Altered nuclear morphologies such as enlargement and increased numbers of nucleoli are observed in many types of cancer cells, including CRC. Nucleoli are sites where *rDNA* transcription and ribosome biogenesis occur.³ To produce a functional ribosome, the precursor rRNA precursor 45s ribosomal RNA (*pre-45s rRNA*) (also known as *47S rRNA*) has to be transcribed from *rDNA* and processed to generate 18 S, 5.8 S and 28 S *rRNAs*.⁴ To support the growth of tumor cells, the rate of ribosomal biogenesis is increased to produce ribosome that is composed of *rRNAs* and proteins, resulting in a prominent increase in nucleolus size.^{5–7} Enhanced proliferation in colon cancer cells should be supported by elevated metabolic mechanisms. To synthesize effectively enough cellular components for cell division, the efficiency of protein synthesis should be enhanced. Increase in the number of ribosomes is one way to support it. As *rRNAs* are the essential component of ribosome, it is expected that the level of primary *rRNA*, *pre-45s rRNA*, should be high so as to maintain

the efficiency of protein synthesis. In this study, we detected the high expression status of *pre-45s rRNA* in two independent cohorts of CRC patients. High expression of *pre-45s rRNA* could be prognostic factor of poor outcome of CRC patients. We explored the underlying mechanism of *pre-45s rRNA* in promoting CRC carcinogenesis and characterized its clinical implication in CRC patients.

RESULTS

Pre-45s rRNA levels are significantly elevated in two cohorts of CRC patients

We first compared the expression level of *pre-45s rRNA* in paired tumor and adjacent normal tissues in two cohorts of CRC patients. We found that 49 out of 52 ($P < 0.0001$) cases from Hong Kong cohort showed higher level of *pre-45s rRNA* in tumor tissues compared with their adjacent non-tumor tissues (Figure 1a); the mean value of relative expression level of *pre-45s rRNA* was also significantly elevated in tumor tissues compared with the adjacent non-tumor tissues ($P < 0.0001$, Figure 1b); however, the expression of *pre-45s rRNA* was not correlated with tumor, node or metastasis (TNM) stages (Figure 1c). The enhanced expression level of *pre-45s rRNA* in tumor tissues was confirmed in the second cohort of 28 pairs of CRC patients from Zhejiang ($P < 0.0001$); the mean expression level of *pre-45s rRNA* was slightly higher in TNM stages

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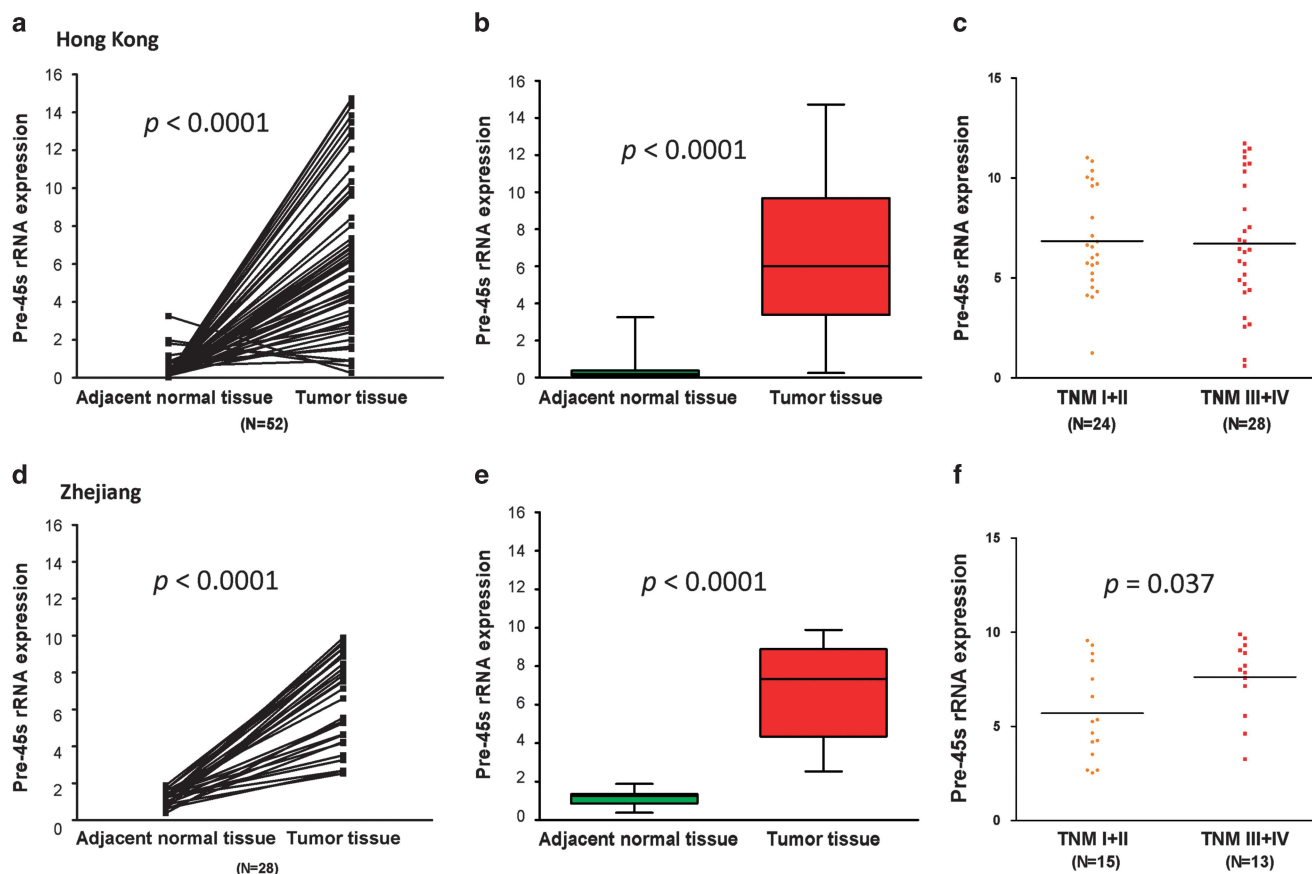


Figure 1. Upregulation of *pre-45s rRNA* in CRC patients. (a) *Pre-45s rRNA* was upregulated in tumor tissues obtained from CRC patients in Hong Kong cohort ($P < 0.0001$, $n = 52$). (b) The average relative expression of *pre-45s rRNA* in both adjacent normal (0.375 ± 0.079) and tumor tissues (6.602 ± 0.563). (c) The expression levels of *pre-45s rRNA* in tumor tissues from the patients at TNM I and II were compared with that from patients at TNM III and IV ($P = 0.867$). (d) *Pre-45s rRNA* was upregulated in tumor tissues obtained from CRC patients in Zhejiang cohort. The expression level of *pre-45s rRNA* in paired adjacent normal and tumor tissues was compared ($P < 0.0001$, $n = 28$). (e) The average relative expression of *pre-45s rRNA* in adjacent normal (1.151 ± 0.068) and tumor tissues (6.591 ± 0.465). (f) The expression levels of *pre-45s rRNA* in tumor tissues from the patients at TNM I and II were compared with that from patients at TNM III and IV ($P = 0.037$). β -Actin was used as an internal control for real-time PCR. Data are expressed as mean \pm s.d.

III/IV compared with stages I/II (Figures 1d–f). Altogether, we confirmed that the expression level of *pre-45s rRNA* in primary tumor tissues was higher compared with that in non-tumor tissues.

Expression level of *pre-45s rRNA* is essential for the growth of colon cancer cells

We evaluated the expression status of *pre-45s rRNA* in nine different colon cancer cell lines (Caco-2, DLD-1, HCT116, HT29, LOVO, LS180, SW480, SW620 and SW1116) and a normal colon epithelial cell line NCM460. All nine colon cancer cell lines showed higher expression of *pre-45s rRNA* relative to NCM460 (Figure 2a). We therefore investigated the functional significance of *pre-45s rRNA* in colon cancer. Downregulation of *pre-45s rRNA* by *pre-45s rRNA siRNA* in three colon cancer cell lines HCT116, HT29 and Caco-2 was confirmed by quantitative reverse transcription–polymerase chain reaction (Supplementary Figures 1A–C). Knockdown of *pre-45s rRNA* significantly suppressed cell growth in all these cell lines (Figure 2b), but not in normal epithelial cells (NCM460; Supplementary Figures 2A–C), suggesting that *pre-45s rRNA* is essential in colon tumorigenesis.

Knockdown of *pre-45s rRNA* inhibits G1/S transition by inducing MDM2/RpL11 interaction and therefore p53 stabilization

To determine whether knockdown of *pre-45s rRNA* decreased colon cancer cell growth by altering cell cycle, we investigated the

effect of *pre-45s rRNA* knockdown on cell-cycle distribution. Downregulation of *pre-45s rRNA* suppressed G1/S transition in colon cancer cells HCT116, HT29 and Caco-2 (Figures 2c–e and Supplementary Figure 2D). Bromodeoxyuridine labeling assay confirmed that knockdown of *pre-45s rRNA* can reduce the portion of cells in the S phase in colon cancer cells HCT116, HT29 and Caco-2 (Supplementary Figure 3). It is well established that cell-cycle arrest can be induced by the overexpression of p53.⁸ Ribosomal proteins can interact with E3 ubiquitin-protein ligase murine double minute 2 (MDM2), suppressing its function on p53 degradation. Among the ribosomal proteins, RpL11 has a significant role in p53 regulation.⁹ We found that downregulation of *pre-45s rRNA* favored the interaction of MDM2 and RpL11 in HCT116 and HT29 cells (Figure 2f) as determined by co-immunoprecipitation, which subsequently resulted in p53 stabilization and upregulation of p53 downstream effectors p21 and p27 (Supplementary Figure 4).

Intracellular Ca^{2+} level mediates upregulation of *pre-45s rRNA* in colon cancer cells

To identify a messenger molecule involved in *pre-45s rRNA* upregulation in colon cancer cells, we tested if cyclic adenosine 3', 5'-monophosphate (cAMP), nitric oxide or calcium ion (Ca^{2+}) would be involved. Our data showed that only treatment with EGTA-AM (a chelator of Ca^{2+}), but not H89 (a protein kinase A

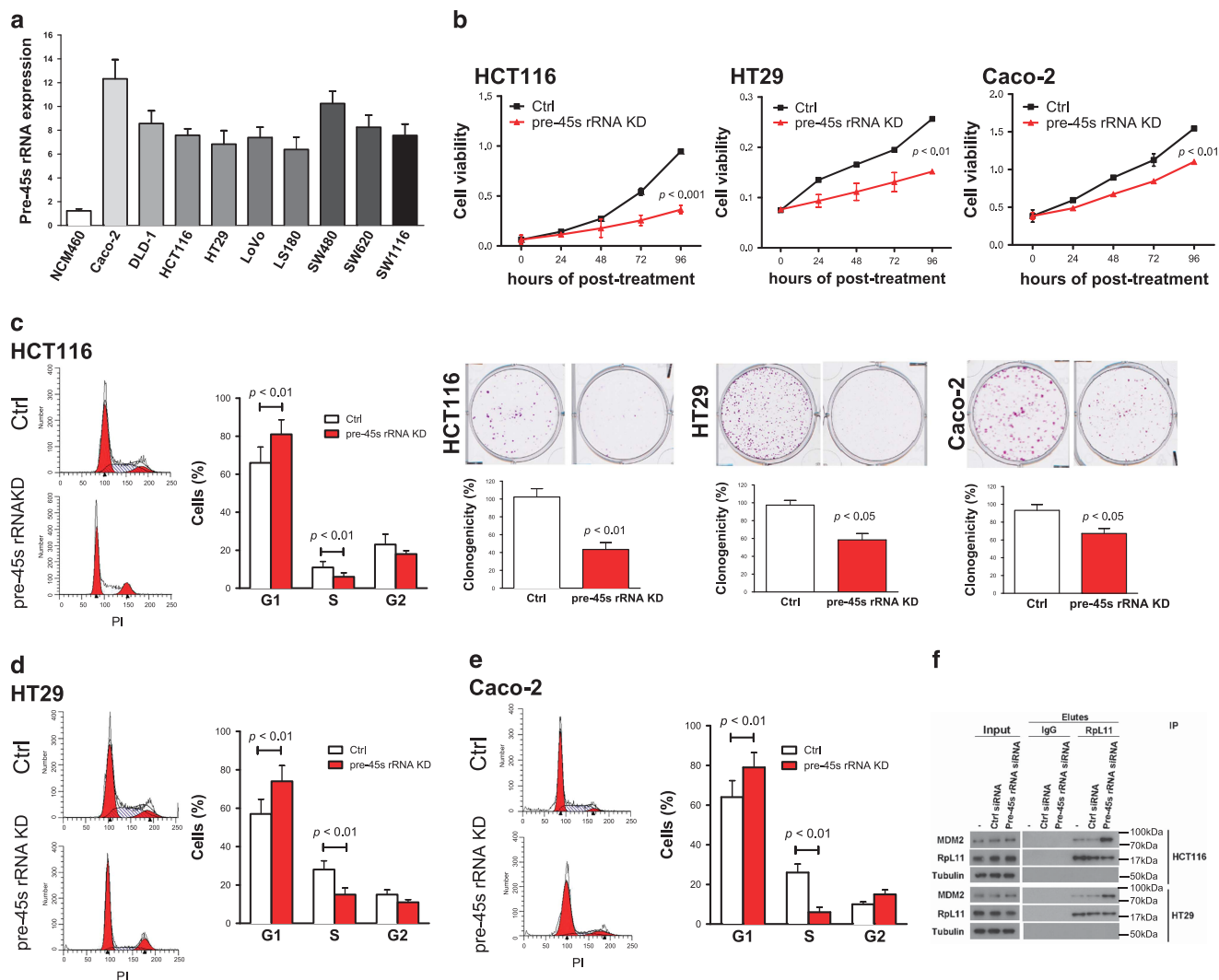


Figure 2. *Pre-45s rRNA* was essential for supporting the growth of CRC tumor cells. (a) Expression levels of *pre-45s rRNA* in colon cancer cell and normal colon epithelial cell were evaluated by real-time PCR. Expression of *pre-45s rRNA* was compared with commercial normal colon tissue. Knockdown of *pre-45s rRNA* suppressed cell growth. (b) MTT assay was performed in HCT116, HT29 and Caco-2. Colony formation assay was performed in HCT116, HT29 and Caco-2. Represented images were shown. Flow cytometry was used to evaluate the effect of *pre-45s rRNA* downregulation on cell-cycle arrest in (c) HCT116, (d) HT29 and (e) Caco-2. Representative graphs were shown. (f) Knockdown of *pre-45s rRNA* favored the interaction between MDM2 and Rpl11. Co-immunoprecipitation was used to determine the interaction between MDM2 and Rpl11. Rpl11 was immunoprecipiated; immunoblot was used to determine the presence of Rpl11 and MDM2 in the immunoprecipitant. siRNA of 5 pmol against *pre-45s rRNA* was used to knockdown the *rRNA* in cells. β -Actin was used as an internal control for real-time PCR. All data points are expressed as mean \pm s.d.

inhibitor) and L-NMMA (an inhibitor of all NOS isoforms), could reduce the level of *pre-45s rRNA* in HCT116 and HT29 colon cancer cell lines (Figure 3). In keeping with this, the concentration of Ca^{2+} was higher in all colon cancer cells compared with normal colon cell line NCM460 (Figure 4a). The feature suggested that the higher concentration of Ca^{2+} might be responsible for mediating the high level of *pre-45s rRNA* in these cells. To verify the relation between Ca^{2+} and *pre-45s rRNA*, we treated nine colon cancer cell lines with another Ca^{2+} chelator, named 1,2-bis(o-aminophenoxy) ethane-*N,N,N,N*-tetraacetic acid (BAPTA-AM), at an optimal dosage of 5 μ M (Supplementary Figures 5A and B). BAPTA-AM significantly reduced the expression of *pre-45s rRNA* in all nine colon cancer cell lines (Figure 4b). However, when these cells were treated with cell-impermeable BAPTA, *pre-45s rRNA* expression did not change (Figure 4b). To further confirm this result, we used Northern blot to determine the expression levels of *pre-45s rRNA* transcript in HCT116 colon cancer cell line. BAPTA-AM treatment reduced the level of *pre-45s rRNA* transcript, whereas BAPTA

treatment did not change *pre-45s rRNA* transcript in HCT116 cells by Northern blot (Supplementary Figure 6A). Therefore, intracellular Ca^{2+} was essential for mediating the upregulation of *pre-45s rRNA*.

CaMKII-56K-UBF axis mediates the upregulation of *pre-45s rRNA*
It is known that CaMKII can be activated by Ca^{2+} in nanomolar level.¹⁰ Activation of CaMKII by Ca^{2+} induces the activity of S6K. The activated S6K subsequently activates upstream binding factor (UBF),¹¹ enhancing the activity of UBF to induce *rRNA* transcription.¹² With these involved connecting components, Ca^{2+} links to *rDNA* transcription. We thus determined the levels of p-CaMKII, p-S6K and p-UBF in colon cancer cells and found that protein expression of p-CaMKII, p-S6K and p-UBF were higher in colon cancer cell lines (HCT116 and HT29) than in normal colon epithelial cell NCM460 (Figure 4c). The expression level of *pre-45s rRNA* was significantly reduced by a CaMKII inhibitor KN93 in colon

cancer cell lines HCT116 and HT29, but not in normal colon epithelial cell NCM460 (Figure 4d). This was confirmed by Northern blot that KN93 could significantly reduce the level of *pre-45s rRNA* level in HCT116 cells (Supplementary Figure 6B).

KN93 treatment reduced the levels of p-CaMKII, p-S6K and p-UBF in HCT116 cells (Supplementary Figure 7A), suggesting that KN93 inhibits CaMKII-S6K-UBF axis. Since the level of *pre-45s rRNA* was essential for the growth of cancer cells, both cell viability and

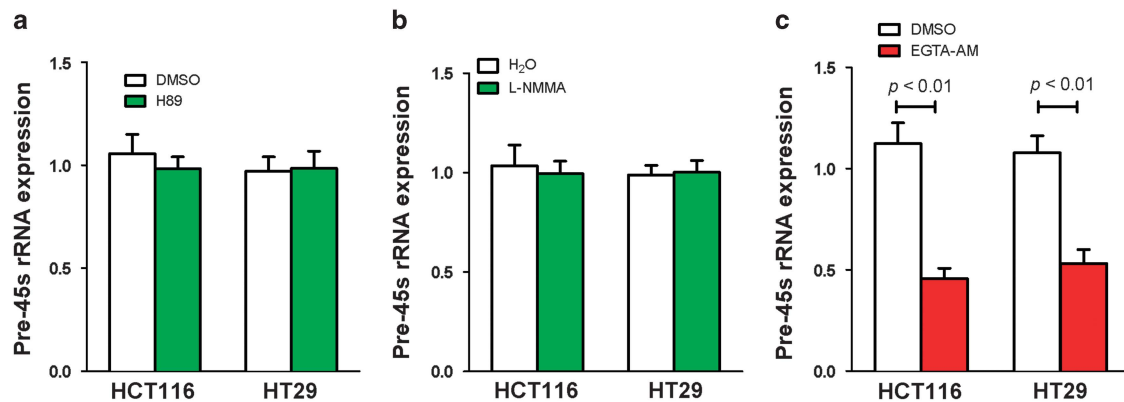


Figure 3. The effect of different chemical on *pre-45s rRNA* level. (a) Cells were treated with 0.1 μM of H89. (b) Cells were treated with 2 μM of L-NMMA. (c) Cells were treated with 1 mM of EGTA-AM. Real-time PCR was performed to test the expression of *pre-45s rRNA*. β -Actin was used as an internal control for real-time PCR. Data are expressed as mean \pm s.d.

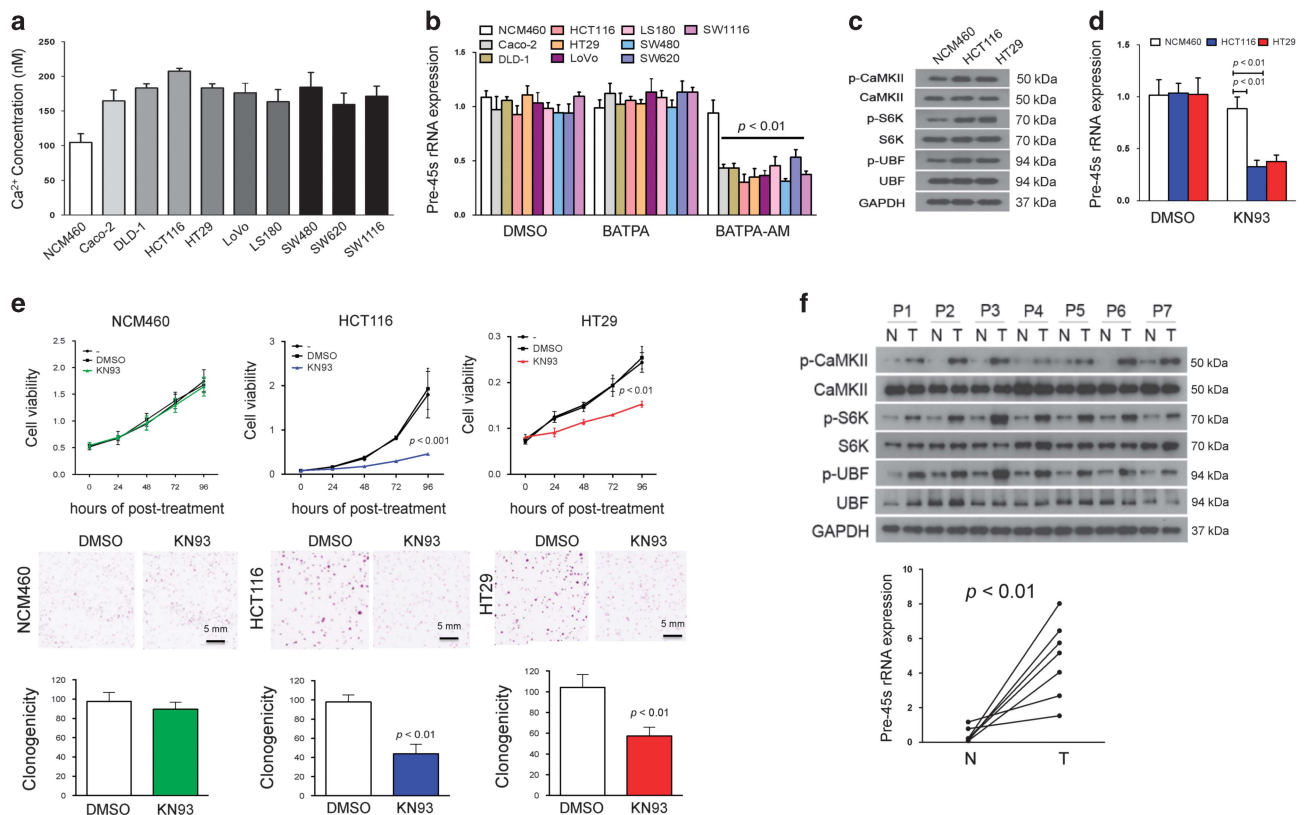


Figure 4. Ca^{2+} used CaMKII-S6K-UBF signaling cascade to mediate high expression level of *pre-45s rRNA* in colon cancer cells. (a) The concentration of free Ca^{2+} in the cytoplasm was determined by fluorescence-based method in normal colon epithelial and colon cancer cells. (b) Treatment of Ca^{2+} chelator BATPA-AM could suppress the expression level of *pre-45s rRNA* in all colon cancer cells. Real-time PCR was used to determine the expression level of *pre-45s rRNA*. (c) Immunoblot analysis on the levels of indicated candidates. (d) Treatment of KN93 suppressed the expression level of *pre-45s rRNA* in colon cancer cells. KN93 is a small molecule that can inhibit the activity of CaMKII. Real-time PCR was performed to determine the level of *pre-45s rRNA*. (e) The effect of KN93 on cell growth in NCM460, HCT116 and HT29 was revealed by MTT. The effect of KN93 on cell growth in NCM460, HCT116 and HT29 was revealed by colony formation assays. Represented images were shown. Five micro molar of KN93 was used to treat cells. (f) Immunoblot analysis on the levels of indicated candidates and real-time PCR analysis on *pre-45s rRNA* levels in paired normal adjacent (N) and tumor (T) tissues from seven patients. Corresponding siRNA of 5 pmol was used. BAPTA-AM/BAPTA of 5 μM was used. GAPDH was used as a loading control in western blot. β -Actin was used as an internal control for real-time PCR. All data points are expressed as mean \pm s.d.

colony formation assays confirmed this growth-suppressive effect by KN93 treatment on HCT116 and HT29 cells, but not in NCM460 (Figure 4e). Moreover, KN93 could increase the level of p53, p21^{Cip/Waf1} and p27^{Kip1} in HCT116 (Supplementary Figure 7B). To further confirm the involvement of CaMKII-S6K-UBF axis in the mediation of *pre-45s rRNA* in colon cancer, we used small interfering RNA (siRNA) to knockdown UBF in HCT116, HT29 and Caco-2. The knockdown efficiency was shown in Supplementary Figure 8A. Knockdown of UBF in these colon cancer cells could reduce the expression level of *pre-45s rRNA* (Supplementary Figure 8B). Clonogenicity assay showed that knockdown of UBF could suppress cell proliferation in HCT116, HT29 and Caco-2 cells (Supplementary Figure 8C). Subsequently, we evaluated the activity of this signaling cascade in primary tumor tissues from CRC patients. The results showed that the signaling cascade was more active in tumor tissues in all cases compared with their adjacent non-tumor tissues, which was consistent with the upregulated *pre-45s rRNA* levels in these tumor tissues (Figure 4f). Collectively, our findings suggested that CaMKII-S6K-UBF axis mediates the upregulation of *pre-45s rRNA* in colon cancer cells.

Loss of function of APC enhances *pre-45s rRNA* expression via inducing CaMKII-S6K-UBF axis

One well known cancer driver in CRC is adenomatous polyposis coli (APC) loss of function. CaMKII-S6K-UBF axis mediated the upregulation of *pre-45s rRNA* would be a general mechanism to support colon tumorigenesis. Therefore, we evaluated the effect of APC knockdown on HCT116 and HT29. HCT116 bears wild type and full length of APC, whereas HT29 bears truncated APC, which is still functional. Knockdown of APC enhanced protein synthesis in these cell lines (Figure 5a). Also, we found that knockdown of APC enhanced the level of free Ca²⁺ in these cell lines (Figure 5b). The increased Ca²⁺ by knockdown of APC could enhance the activity of CaMKII-S6K-UBF axis, whereas the enhanced activity of this signaling cascade could be blocked by BAPTA-AM in both HCT116 and HT29 (Figure 5c). As a result, knockdown of APC led to the elevation of *pre-45s rRNA* level, whereas such an elevation could be abolished by BAPTA-AM (Figure 5d). Moreover, the enhanced cell growth induced by APC knockdown could be diminished by knockdown of *pre-45s rRNA* in both colon cancer cells (Figures 5e and f). However, there is no significant difference of *pre-45s rRNA* levels in HCT116 p53^{+/+} and HCT116 p53^{-/-} cells (Supplementary Figure 9). These data indicated that *pre-45s rRNA* is important in supporting tumorigenesis mediated by certain CRC drivers such as APC.

High expression of *pre-45s rRNA* is an independent predictor of poor outcome in CRC patients

The association between clinicopathologic features and *pre-45s rRNA* expression in human CRCs was evaluated. We categorized the patients into two groups, based on the expression level of *pre-45s rRNA*, high expression (\geq mean in the study cohort) and low expression ($<$ mean) groups. *Pre-45s rRNA* expression only associated with tumor length and recurrence in Hong Kong cohort (Table 1). We found a positive and linear relationship ($R^2 = 0.8232$) between these tumor lengths and *pre-45s rRNA* level with Spearman's coefficient equaled to 0.9163 (Figure 6a). Therefore, our data implicated that *pre-45s rRNA* might be one of factors to support CRC carcinogenesis. We next examined that correlation between the expression level of *pre-45s rRNA* and survival outcome in CRC patients. In both Hong Kong cohort and Zhejiang cohort, the survival outcomes were significantly different between high expression and low expression groups as shown in the Kaplan–Meier survival curves ($P < 0.0001$; Figures 6b and c). We further used univariate and multivariate Cox regression analyses to evaluate the disease-free hazard ratio (HR) of different parameters

in the two cohorts. We found that the survival outcomes were significantly different between high *pre-45s rRNA* expression and low *pre-45s rRNA* expression groups (univariate: HR = 5.941; 95% confidence interval (CI) = 2.924–12.070; multivariate: HR = 7.385; 95% CI = 3.504–15.568; Table 2). Therefore, high expression level of *pre-45s rRNA* was an independent prognostic factor for poor outcome of CRC patients.

DISCUSSION

In this study, we identified that *pre-45s rRNA* was markedly upregulated in primary tumors of CRC and colon cancer cell lines. A series of *in vitro* functional experiments revealed that *pre-45s rRNA* possessed a strong tumorigenic function in CRC. *Pre-45s rRNA* enhanced tumor cell proliferation, which is attributable to its G1/S cell-cycle-promoting ability. The mechanism of the upregulation of *pre-45s rRNA* in colon cancer cells was investigated and we found that higher concentration of Ca²⁺ was involved in mediating the increased level of *pre-45s rRNA* in CRC. Changing the cytosolic concentrations of Ca²⁺ leads to a broad range of cellular events, including those important in tumorigenesis mediated by specific Ca²⁺ channels and pumps.^{13–15} The high level of *pre-45s rRNA* induced by higher concentration of Ca²⁺ is at least one of the molecular mechanisms contributing to the tumorigenesis of CRC (Figure 6d).

Pre-45s rRNA can be processed to 18 s and 28 s rRNA for assembly of large and small ribosome subunits. Ribosomal proteins of Rpl5, Rpl11 and Rpl23 are essential elements of ribosome subunits. It was reported that inhibition of rRNA by nucleolar stress led to the accumulation of free ribosomal proteins including Rpl5, Rpl11 and Rpl23 Rpl23.^{16–18} The unassembled free Rpl11 could interact with MDM2 and led to a marked accumulation of p53.¹⁹ In this study, we found that knockdown of *pre-45s rRNA* enhanced MDM2/Rpl11 interaction (Figure 2f). Collectively, these findings suggested that depletion of *pre-45s rRNA* enhances MDM2/Rpl11 interaction through the release of free Rpl11, thereby inhibiting colon cancer cell proliferation.

We further extend the knowledge on roles played by non-ATP-dependent channels in colon cancer cells. We found that high metabolism could lead to increase in cytosolic Ca²⁺ via translocons. We confirmed this notion by treating cells with protein synthesis inhibitors cyclohexamer and anisomycin. Through gene knockdown and pharmaceutical intervention (CaMKII inhibitor KN93) approaches, we uncovered CaMKII-S6K-UBF axis for mediating the upregulation of *pre-45s rRNA* in colon cancer cells (Figure 6d). It was reported that inhibition of Ca²⁺ by an N-myristoylated EF-hand Ca²⁺-binding protein, calcineurin homologous protein 1, suppresses rRNA transcription by interacting with UBF, which is a component of the RNA polymerase I complex.²⁰ Inhibition of RNA polymerase I promotes the cancer-specific activation of p53 and subsequent suppression of tumor growth.²¹ On the other hand, the interaction between calcineurin homologous protein 1 and UBF can be abolished by the presence of Ca²⁺, thereby promoting rRNA transcription.^{20,22} These findings collectively suggested that the effect of CaMKII-S6K-UBF on the regulation of *pre-45s rRNA* transcription in CRC is potentially mediated by RNA polymerase I complex.

Mutations on APC activate Wnt signaling pathway,^{23–25} resulting in enhanced protein synthesis.^{6,26} In noncanonical Wnt β -catenin-independent signaling pathway, activation of Wnt signaling can induce the elevation of Ca²⁺ in the cytoplasm and subsequent activation of CaMKII.²⁷ The binding of Wnt to Frizzled receptor triggers the production of inositol 1,4,5-triphosphate and 1,2 diacylglycerol.²⁸ Both inositol 1,4,5-triphosphate and 1,2 diacylglycerol can induce the release of Ca²⁺ through the interaction with the calcium channels present on the endoplasmic reticulum membrane.²⁷ Sustained activation of Wnt signaling maintains a relative high intracellular Ca²⁺, which induces the activation of

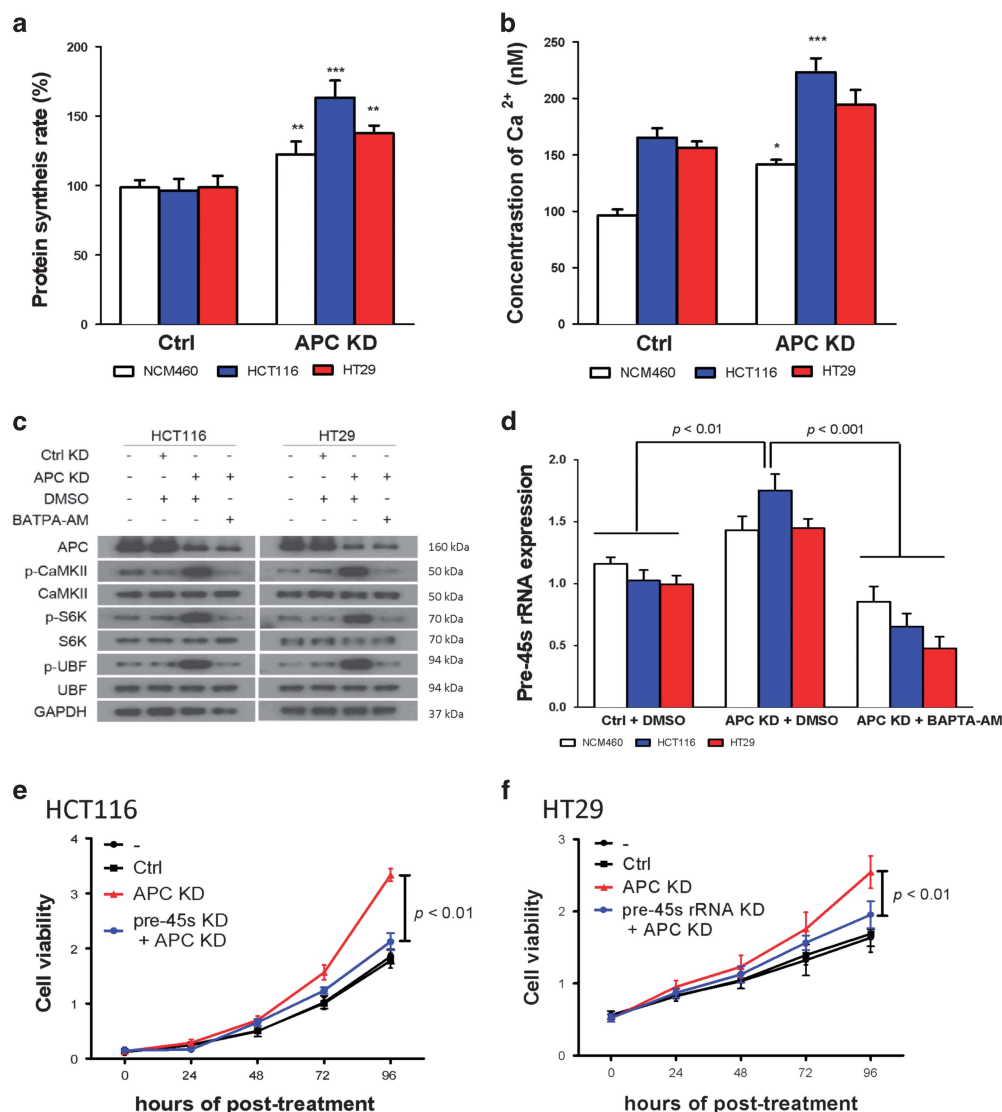


Figure 5. *Pre-45s rRNA* was essential for supporting the cell growth in colon cancer cells with APC loss of function. **(a)** Knockdown of *APC* enhanced the rate of protein synthesis in colon cell lines. **(b)** Knockdown of *APC* led to elevation of free Ca^{2+} in the cytoplasm in colon cells. Real-time PCR was used to determine the expression level of *pre-45s rRNA*. **(c)** Activation of CaMKII-S6K-UBF signaling cascade in *APC* knocked down cells depended on free Ca^{2+} . GAPDH was used as a loading control. Cells were treated with 5 μ M of BATPA-AM. **(d)** Upregulation of *pre-45s rRNA* mediated by *APC* knockdown was abolished by BATPA-AM treatment in colon cancer cells. Expression of *pre-45s rRNA* was compared with untreated NCM460, HCT116 and HT29. Knockdown of *pre-45s rRNA* suppressed cell growth in colon cancer cells. **(e)** HCT116 and **(f)** HT29 with *APC* knocked down. Cell growth was evaluated by MTT assay. siRNA of 5 pmol against *pre-45s rRNA* was used to knockdown the *rRNA* in cells. GAPDH was used as a loading control in western blot. β -Actin was used as internal control for real-time PCR. All data points are represented as mean \pm s.d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

CaMKII-S6K-UBF axis and eventually leading to high expression of *pre-45s rRNA*. In addition, we found that knockdown of *APC* enhanced *pre-45s rRNA* levels, and consequently promoted cell growth. On the other hand, downregulation of *pre-45s rRNA* abolished the promoting effect of *APC* knockdown on cell growth. These findings suggest that CRC driver mutation such as *APC* also contributed to the upregulation of *pre-45s rRNA* to sustain their oncogenic function in CRC. Thus, *pre-45s rRNA* is a central hub of CRC carcinogenesis.

Recognizing the biological functions of *pre-45s rRNA* in CRC, its enhanced expression would favor tumor progression and a worse outcome in the patients. In this regard, the clinical significance of *pre-45s rRNA* expression with patient's outcome was evaluated in two independent cohorts of 80 primary CRC patients. Our results indicated that higher *pre-45s rRNA* expression

was significantly associated with poor survival in cohort I (Hong Kong) CRC patients independent of patient characteristics ($P < 0.0001$). This finding was supported by that of cohort II (Zhejiang; $P < 0.0001$). Our data supported an adverse effect of *pre-45s rRNA* on the survival of CRC patients, providing an additional evidence for the promoting role of *pre-45s rRNA* in the development of CRC.

In conclusion, we demonstrated that *pre-45s rRNA* is upregulated in tumor tissues and such an upregulation is mediated by signaling cascade composed of CaMKII-S6K-UBF mediated by Ca^{2+} . The high level of *pre-45s rRNA* level promotes the development of CRC by inducing G1/S cell-cycle transition through suppressing MDM2/RpL11 interaction and therefore reducing p53 stabilization (Figure 6d). *Pre-45s rRNA* is associated with the poor survival of CRC patients.

Table 1. The association between expression level of *pre-45s rRNA* and clinical parameters in two cohorts of patients with CRC

Variables	Hong Kong cohort		Zhejiang cohort		
	No. of patients	P-value	No. of patients	P-value	
Age (years)					
≥ 66	25	0.068	≥ 50	25	0.073
< 66	27		< 50	3	
Gender					
M	35	0.419	M	21	0.0787
F	17		F	7	
TNM stage					
I/II	24	0.634	I/II	15	0.360
III/IV	28		III/IV	13	
Tumor length (mm)					
> 3	35	< 0.001	N/A		
≤ 3	17				
Recurrence					
Y	24	< 0.001	N/A		
N	28				
<i>Pre-45s rRNA</i> expression level					
High	25		High	19	
Low	27		Low	9	

Abbreviations: CRC, colorectal cancer; N/A, not applicable; TNM, tumor, node, metastasis.

MATERIALS AND METHODS

Clinical samples collection and study

Eighty pairs of CRC tumor and their adjacent non-tumor tissues were collected from 1999 to 2009 who underwent surgery at The Prince of Wales Hospital, Hong Kong ($n=52$) and The Second Affiliated Hospital, Zhejiang ($n=28$), and were histologically confirmed in a blinded manner by two pathologists. The adjacent normal tissue is composed of normal colonic mucosa located ~10 cm away from the cancer tissue. All subjects provided informed consent before specimen collection. The study protocols were approved by the Ethics Committee of the Chinese University of Hong Kong and Human Subject Research Ethics Committee of the Second Affiliated Hospital, School of Medicine, Zhejiang University.

Cell cultures

Colon cell lines (Caco-2, DLD-1, HCT116, HT29, LOVO, LS180, SW480, SW620 and SW1116) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). NCM460 was obtained from In Cell (San Antonio, TX, USA). HCT116 p53^{-/-} was provided by Dr B Vogelstein (Johns Hopkins University, Baltimore, MD, USA). Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% of fetal bovine serum and 1% of penicillin and streptomycin was used.

RNA extraction and real-time PCR analyses

Total RNA was extracted from cells, tissues and serum using TRIzol Reagent (Molecular Research Center Inc., Cincinnati, OH, USA). cDNA was synthesized from 2 µg of total RNA using Transcriptor Reverse Transcriptase (Roche, Indianapolis, IN, USA). β -Actin was used as internal control; the primer sequences for β -actin were as follows: forward, 5'-AAAAGCCACCCCACTTCTCT-3' and reverse, 5'-CTCAAGTTGGGGGACAAAAA-3'. The primer sequences of *pre-45s rRNA* were as follows: forward, 5'-CCGTCCGTCGGTCGTCCTCTCGC-3' and reverse, 5'-TGTACCGCCGTGCG TACTTA GAC-3'. Real-time PCR was performed using the SYBR Green master mixture (Roche, Indianapolis, IN, USA) on the ABI7500 Instrument (Thermo Fisher Scientific, Grand Island, NY, USA). Each sample was tested in triplicate. $\Delta\Delta$ CT method was used to determine the fold change in gene expression level. Δ CT method was used to determine the relative expression levels of corresponding genes.

RNA interference and transfection

Knockdown of *pre-45s rRNA* expression in was performed by siRNA targeting *pre-45s rRNA* (5'-UCAACCCAAGGACACACGA[dT][dT]-3' (Sigma-Aldrich, St Louis, MO, USA). HCT116, HT29 and NCM460 cells were transfected with 50 nM APC siRNA (s1434; Life Technologies, Carlsbad, CA, USA), CaMKII siRNA (L-004536; Dharmacon, Lafayette, CO, USA), ribosomal S6K siRNA (L-003616; Dharmacon), UBF siRNA (sc-29514, Santa Cruz Biotechnology, Dallas, TX, USA) or control siRNA (AM4611) using Lipofectamine 2000 (Life Technologies).

Chemicals

KN93 (Santa Cruz Biotechnology), H89 (Sigma-Aldrich), L-NMMA (Cayman Chemical Company, Ann Arbor, MI, USA), EGTA-AM (Life Technologies), BAPTA-AM (Sigma-Aldrich) and BAPTA (Sigma-Aldrich) were purchased. These chemicals were dissolved in dimethyl sulfoxide or double-distilled water.

Western blot analysis

Total protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The proteins in sodium dodecyl sulfate–polyacrylamide gel electrophoresis were transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The membrane was incubated with primary antibodies overnight, and then with secondary antibody at room temperature for 1 h. Proteins of interest were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare). The antibodies used and their dilutions were listed in Supplementary Table S1.

Northern blot

DNA probe against *pre-45s rRNA* 5'-CTGACACGCTGTCTCTGGCGA-3' and the probe against actin 5'-TAGGATGGCAAGGGACTTCTG-3' were used. The probes were biotin-labeled. The RNA sample was denatured by heating at 65 °C for 15 min in 50% of formamide. Ten micrograms of denatured RNA was resolved by electrophoresis in 4% of denaturing polyacrylamide gel. RNA in the gel was then transferred to Nylon membrane Hybond-N+ (GE Healthcare). RNA was crosslinked to the membrane by UV illumination. Signal was generated by using North2South Chemiluminescent Hybridization and Detection Kit (Life Technologies). X-ray film was used to show the signal.

Cell viability assay

Cell viability was examined using the Vybrant MTT Cell viability Assay Kit (Life Technologies) according to the manufacturer's instructions. All experiments were conducted three times in triplicates. Bromodeoxyuridine (B23151) and Bromodeoxyuridine Monoclonal Antibody Alexa Fluor 488 (B35130) were purchased from Life Technologies. The labeling and detection were performed according to the manufacturer's protocol.

Protein synthesis measurement

Click-iT HPG Alexa Fluor 488 Protein Synthesis Assay Kit was purchased (Life Technologies). Experiments were performed according to manufacturer's instructions. All experiments were conducted three times in triplicates.

Colony formation assay

Cells (2×10^5 /well) were plated in a 12-well plate. After 24 h, cells were treated with indicated treatment. After culturing for 4 to 12 days, cells were fixed with 70% ethanol and stained with 0.5% crystal violet solution. Colonies with more than 50 cells per colony were counted. All experiments were conducted three times in triplicates.

Cell-cycle analysis

The cells (HCT116, HT29, Caco-2 and NCM460) were fixed in 70% ethanol-phosphate-buffered saline for 24 h. The cells were then labeled with 50 µg/ml of propidium iodide (BD Pharmingen, Franklin Lakes, NJ, USA). The cells were sorted by FACSCalibur (BD Biosciences, San Diego, CA, USA). Cell-cycle profiles were analyzed by the ModFit 3.0 Software

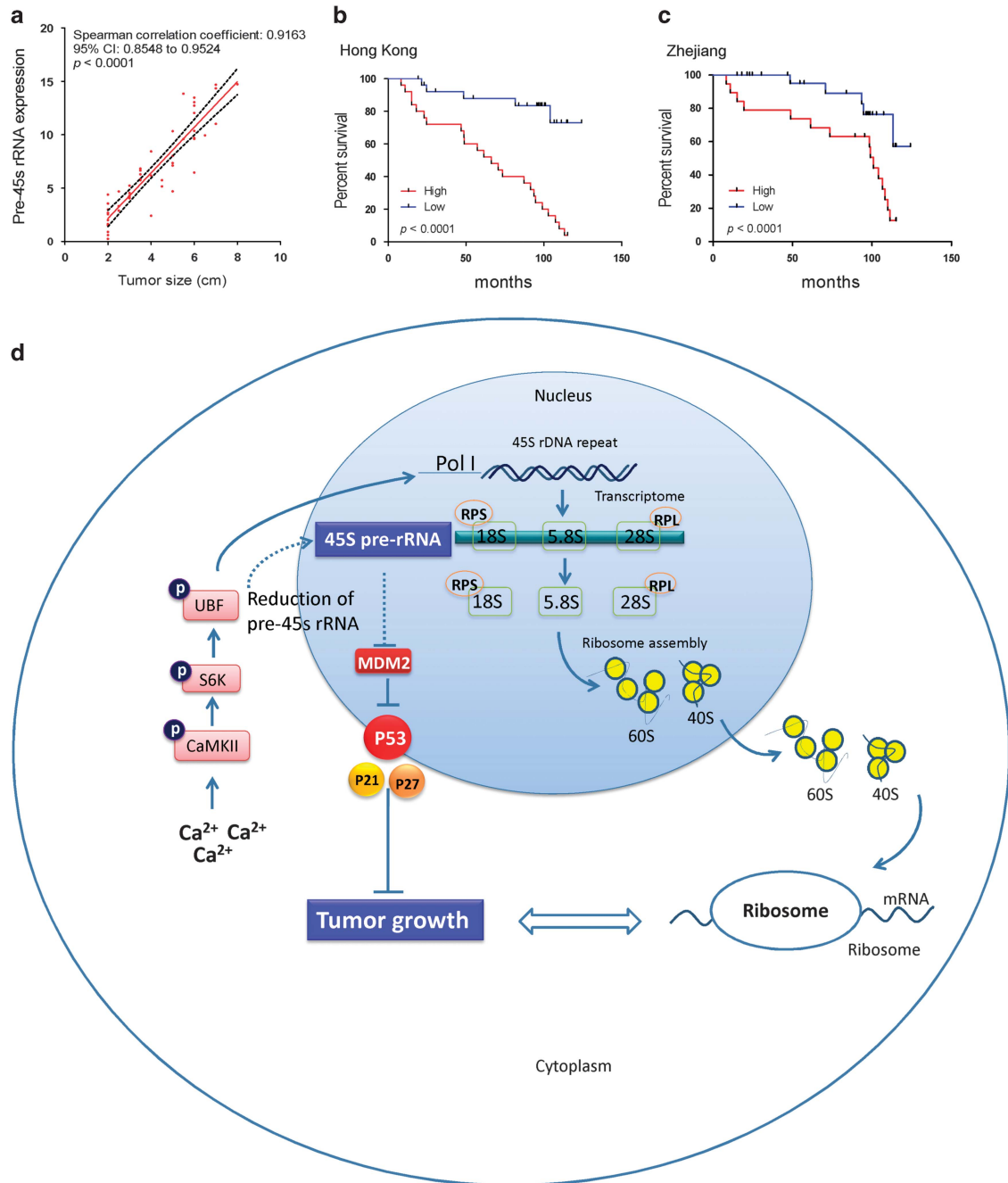


Figure 6. Clinical significance of *pre-45s rRNA* in CRC patients. (a) Tumor size positively correlated with the expression level of *pre-45s rRNA*. Only samples from Hong Kong were included in this analysis. Red line represents 95% CI. High expression level of *pre-45s rRNA* correlated with poor survival outcome of patients from (b) Hong Kong and (c) Zhejiang. Expression level of *pre-45s rRNA* was an independent prognostic marker for CRC patients. (d) Schematic illustration of the molecular mechanism of *pre-45s rRNA* in colon cancer. The high metabolism in the colon cancer cells triggers the passive release of Ca^{2+} from endoplasmic reticulum to the cytoplasm. The elevated Ca^{2+} in the cytoplasm activates the signaling cascade composed of CaMKII-S6K-UBF. The activated UBF promotes the transcription of rDNA by different mechanisms, which therefore increases the level of precursor of ribosomes, *pre-45s rRNA*. The enhanced *pre-45s rRNA* promotes G1/S cell-cycle transition by reducing the inhibitory factors on MDM2 and therefore decreasing the level of p53, which contributes to colon tumorigenesis.

(BD Biosciences). All experiments were conducted three times in triplicates.

Ca²⁺ measurement

Cells were treated with 4 μ M of Fura-2-AM (Life Technologies) for 10 min. Fluorescence intensity was measured by Perkin-Elmer Fluorescence Spectrometer (Perkin-Elmer, Waltham, MA, USA). Standard curve was

plotted for calculating the concentration of Ca^{2+} . All experiments were conducted three times in triplicates.

Statistical analysis

The difference in *pre-45s rRNA* levels between paired tissue samples was determined by the Wilcoxon's matched-pair test. Correlations between tumor size and *pre-45s rRNA* expression level were determined by the

Table 2. Cox regression analysis of a potential poor outcome predictor for patients with colon cancer in Hong Kong and ZheJiang

Variables	Univariate Cox regression			Multivariate Cox regression		
	HR (disease-free)	95% CI	P-value	HR (disease-free)	95% CI	P-value
Age						
≥66 (n = 25)	1.236	0.669–2.284	0.499			
< 66 (n = 27)	1					
Gender						
Male (n = 35)	1.375	0.706–2.679	0.349			
Female (n = 17)	1					
TNM stage						
I/II (n = 24)	1			1		
III/IV (n = 28)	3.400	1.789–6.462	< 0.001	4.430	2.224–8.824	< 0.001
Pre-45s rRNA expression						
High (n = 27)	5.941	2.924–12.070	< 0.001	7.385	3.504–15.568	< 0.001
Low (n = 25)	1			1		

Abbreviations: CI, confidence interval; HR, hazard ratio; TNM, tumor, node, metastasis.

Spearman's correlation test. Disease-free HR of survival associated with *pre-45s rRNA* level and other predictor variables were first estimated using Univariate Cox proportional hazards regression model. Multivariate Cox model was constructed to estimate the adjusted HR for high expression of *pre-45s rRNA*. $P < 0.05$ was taken as statistical significance. All the tests were performed by the Graphpad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) or SPSS18 (IBM Corporation, Armonk, NY, USA).

ABBREVIATIONS

ATP, adenosine triphosphate; APC, adenomatous polyposis coli; BAPTA, 1,2-bis(o-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid; cAMP_r, cyclic adenosine 3', 5'-monophosphate; Ca²⁺, calcium ion; CaMKII, calcium/calmodulin-dependent protein kinase II; CRC, colorectal cancer; HR, hazard ratio; MDM2, mouse double minute 2 homolog; pre-45s rRNA, precursor 45s ribosomal RNA; Rpl11, ribosomal protein L11; S6K, ribosomal S6 kinase; UBF, upstream binding factor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HT designed the study, performed the experiments, analyzed data and drafted the manuscript. KCL, YD, CKL and JZ performed experiments. SCN, SSMN, YXC, SZ and JF collected samples. XZ analyzed data and revised the manuscript. JY designed, supervised the studies and wrote the paper.

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