Early-stage colon cancer with high MALAT1 expression is associated with the 5-Fluorouracil resistance and future metastasis

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Abstract

Background: This study aimed to investigate the role of long noncoding RNA (LncRNA) expression profiles to predict relapse and 5-FU response in patients with stage I/II colon cancer (CC).

Methods and Results: The expression level of 15 LncRNA was analyzed in stage I/II colon tumors of 126 CC patients. To confirm the findings in-vitro, 5FU-resistant HT29 cells were generated by subjecting HT-29 cells to the increasing concentrations of 5FU for 6 months. The 5FU resistance was observed in WST-1 and Annexin V analyses. The colony formation and wound healing assays were assessed to determine the metastatic properties of the cells. Expression levels of LncRNAs and mRNA of EMT-related genes were determined by RT-PCR. The role of LncRNA on metastasis and 5FU sensitivity were confirmed in pcDNA3.0-PTENP1 and si-MALAT1 expressed 5FU-resistant HT29 cell lineages.

Results: High MALAT1 (p=0.0002) and low PTENP1 (p= 0.0044) expressions were significantly associated with 5-FU resistance and tumor relapse in stage I/II CC. The invasiveness and colony-forming characteristics of 5-FU-resistant cell lineages were higher as compared to the parent HT-29. Moreover, the expression of MALAT1 (p=0.0009) was increased while the expression of PTENP1 (p=0.0158) decreased in 5FU-resistant-HT-29 cells. Si-MALAT1 treatment increased cell sensitivity to 5FU, whereas it decreased invasive behaviors of 5 FU-resistant-HT-29 cells.

Conclusion: MALAT1 may be a biomarker in predicting recurrence in early-stage CC. Our findings suggest that a cell-based therapy to target MALAT1 could be established for these patients to prevent metastasis and 5-FU resistance.

Introduction

Colon cancer (CC) is one of the most frequent malignant diseases worldwide with a remarkably high mortality rate [1]. In patients with Stage II CC, adjuvant chemotherapy remains controversial. Treatment decisions and prognosis assessment depend on tumor node metastasis staging. However, this staging system cannot predict the risk of recurrence of Stage II CC patients [2]. The National Comprehensive Cancer Network (NCCN) guidelines define the poor prognostic factors of CC as follows: the presence of poorly differentiated histology, presence of lymphovascular invasion, and perineural invasion, reports of less than 12 lymph nodes, intestinal obstruction, localized perforation, presence of mucinous component and stony ring cell structure. Stage I/II colon tumors which have one of these features are defined as at high risk for systemic or local recurrence [3]. Although the histopathological, clinical, and genetic prognostic factors for CC have been widely studied, our understanding of the factors that affect the recurrence risk of patients with different clinical outcomes is largely limited [4,5].

Chemoresistance is the key barrier to the efficacy of CC treatment. The 5-fluorouracil (5FU)-based chemotherapy regimens are therapeutically effective for only 10–16% of advanced CC cases [6]. Besides, the molecular association between metastasis and resistance to 5FU based therapy has not been fully revealed. Although metastasis is a commonly observed event in advanced patients, predicting a future

metastasis potential is difficult for early-stage patients [7]. Therefore, to foresight the long-term effects of adjuvant therapy and to avoid potential metastasis risk, there is a need to identify drug resistance and metastasis-associated biomarkers in CC patients.

Long non-coding RNA (LncRNAs) are approximately 200 nucleotides long, endogenously encoded single-stranded RNAs that are not encoded an mRNA of a protein [8]. LncRNAs are involved in the regulation of the key signaling pathways involved in proliferation, invasion, and apoptosis [9]. Recently, evidence has indicated that LncRNAs can function as oncogenes or tumor suppressor genes and may serve as prognostic and predictive markers in tumor development and progression [10–12]. In CC, LncRNAs are involved in every stage of carcinogenesis and tumor progression including tumor initiation and migration of cancer cells, angiogenesis, tumor invasion, and metastasis formation [13]. However, there are a limited number of studies on the expression profiles and prognostic values of LncRNA in stage II CC [14,15].

Determining the recurrence potential of patients with stage I/II CC is of great importance for these patients to benefit from adjuvant chemotherapy. Therefore, the first aim of this study is to investigate the availability of LncRNAs to predict the potential for liver metastasis in stage I / II colon tumors that are not routinely evaluated for poor prognostic factors. Because poor prognostic factors may also lead to drug resistance, the second aim of our study is to predict the response of tumors with identified recurrence potential to the standard 5FU chemotherapy. For these purposes, first; the LncRNA expression profiling in early-stage CC patients without poor prognostic features was investigated. Second; the importance of expression changes in LncRNAs was determined in-vitro.

Material And Methods

Cohort Analysis

Patients Selection

The present study included 126 unrelated T1-3N0M0 sporadic CC patients who underwent colon resection without neoadjuvant chemo-radiotherapy in xxxxx University Hospital from 1997 to 2012. Of the 126 patients, 69 were male, and 57 were female; the patients had a median age of 62.75 years (range, 19–71 years). Primary tumors were localized in the right colon in 61 cases and left colon in 65 cases. Tumor stages were classified as T1 (n = 11), T2 (n = 36), and T3 (n = 79). All recruited patients received neither chemotherapy nor radiotherapy before the surgery. All patients were sporadic cases. The only stage I and stage II tumors (T1-3N0M0) were included, and the patients could not display the histopathological features associated with poor prognosis, including the presence of a mucinous component or signet ring cells, a lymphatic or vascular invasion, or less than 12 harvested lymph nodes; this aided in creating a homogenous study population. Tumors with systemic recurrence other than liver metastasis were not included in the study. All patients with Stage I / II were followed up for at least 5 years for recurrence. The follow-up after surgery was based on periodical clinical visits and the results of biochemical tests and imaging work-ups. In addition to Stage I/II patient’s tumor tissue sample cohort,

non-tumoral tissues of the same patients were enrolled in the study as the negative control. Besides, forty Stage III (n = 20) and Stage IV (n = 20) patients were enrolled to do the study as positive control tumor tissues. A pathologist confirmed all formalin-fixed and paraffin-embedded (FFPE) tissues, and the areas of the slide representing “tumor” (highest numbers of cancer cells present) and “nontumor” were identified.

RNA analysis and RT-qPCR

The expression level of 15 LncRNAs (MALAT1, CCAT1, HOTAIR, PTENP1, CRNDE, ANRIL, SNHG16, TUSC7, HULC, PCAT1, DANCR, MEG3, NEAT1, BANCR, H19) was evaluated which are associated with metastasis and poor prognosis in tumor samples and controls. For FFPE tissues, total RNA was isolated from the FFPE tumor and normal tissue using the RNeasy FFPE Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer’s protocol, with an additional DNase digestion step and elution of RNA in 15 µl RNase-free water. 5 ng of the total RNA was used for the reverse transcript to cDNA according to instructions of the relative expression of target mRNA levels between the cell lines was compared with the Ct method. GAPDH (Hs04420697_g1) was used as an internal standard.

In-vitro Analysis

Cell culture and reagents

Human colorectal cancer cell line, HT-29 (HTB-38) and Human umbilical vein endothelial cell line, HUVEC obtained from American Type Culture Collection (ATCC). HT-29 and HUVEC cells were cultured in a standard medium containing High glucose Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 15% FBS and 1% penicillin/streptomycin. Cells were maintained in a 5% CO2 humidified incubator at 37°C. 5FU was purchased from Sigma-Aldrich (St Louis, MO, USA).

Establishment of drug resistance HT-29 cell sublines

Using continuous exposure to increasing the 5FU concentration method, HT-29 cells were induced by 5FU with a starting concentration of 0.1 µg/ml to 10 µg/ml as described in the study of Yang and colleagues [16]. Briefly; after cells stably grew, cells were passaged for 2 to 3 generations, and the concentration of 5FU was increased by the following concentration gradient; the induced drug concentration. The drug concentration was gradually increased for 6 months. The five HT-29 sublines that acquired resistance to 5FU were named according to level of resistance (HT-29F1: 0.1 µg/ml-3 weeks; HT-29F2: 0.5 µg/ml-4 weeks; HT-29F3: 1 µg/ml-5 weeks, HT-29F4: 5 µg/ml-6 weeks and HT-29FUR: 10 µg/ml-6 weeks;). HT-29 parental cells transformed to HT-29FUR subline within the 15th passage. The morphological characteristics of the cells were observed using an inverted microscope in x40 magnification (Labomed, USA).

Modification of LncRNA expression in CC cells

To determine the role of MALAT1 and PTENP1 in CC tumourigenesis, drug resistance, and progression, HT-29FUR cells were transfected with pcDNA3.0-PTENP1, si-MALAT1 (5-

CACAGGGAAAGCGAGUGGUUGGUA-3) and their combinations (Shanghai GenePharma Co., Ltd., Shanghai, China) PTENP1 using Lipofectamine 2000 (Invitrogen; Thermo Fischer Scientific, Inc.) according to a standardized protocol. pcDNA3.0 and a noncoding siRNA (si-NC) (5′ UUCUCCGAACGUGUCACGU-3′) were used as negative controls, respectively.

RT-PCR

MALAT1, PTENP1, EMT biomarkers genes (*E-CADHERIN*, *N-CADHERIN*, *SLUG*, *SNAIL*, *ZEB1*, *TWIST*, *MMP-9*, and *VIMENTIN*) and mRNA expression of 88 genes which involved in signaling pathways of colorectal tumorigenesis (ref KEGG) were analyzed in generated 5FU resistance cell lineages of, as well as pcDNA3.0-PTENP1 and si-MALAT1 transfected HT-29 cells using RT-PCR analysis. Total RNA was extracted from the cells with E.Z.N.A." Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA). All RNA samples were assessed for quality using a spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). ProtoScript First Strand cDNA Synthesis Kit (Biolabs, USA) in a thermal cycler (i-Cycler, Bio-Rad Laboratories, USA). Taqman probe-based RT-qPCR reaction was performed according to the manufacturer's protocol (Thermo Fisher, US) using Step One Plus Real-time PCR system (Applied Biosystems, USA). The analysis was carried out in three replicates.

Cell proliferation assay

Five HT-29 sublines, parental cells, and transfected cells which were in the logarithmic growth phase were seeded at 2×10^4 cells per well in a 96-well plate and cultured with 100 µL medium per well. Five different doses of 5FU (0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml) were applied to the cells for 24, 48, and 72 hours. A total of 100 µL medium was used as control. WST-1 proliferation assay (BioVision, San Francisco, CA) was utilized to quantify the cell viability according to the manufacturer's instruction. The experiment was repeated 3 times for each sample. The optical density (OD) at 450 nm was quantified using a multimode microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Annexin V analysis

HT-29 and HT-29FUR cells were treated with 5FU concentrations for 72h and Annexin V assay (Muse Annexin V and Dead Cell Assay Kit; Millipore, Darmstadt, Germany) was assessed according to manufacturer's instruction and analyzed using Muse Cell Analyzer (Millipore). The analysis was carried out three times for each sample.

Wound healing analysis

Wound healing analysis was used to observe the ability of HT-29FUR and transfected cells to migrate capabilities. 750x103 cells were seeded per well in a 6-well plate for this analysis. Cells were incubated until the cell density in each well reached 90%. The cell layer was scratched with a sterile 200 µl pipette tip through the confluent monolayer and washed with PBS to remove cell debris. After application of 5FU with certain doses, the recovery in scratched wounds was observed and photographs were captured at 0th,6th,12th,18th, and 24th hours of incubation using an inverted microscope (Labomed, USA). All

samples were assayed in triplicates. The level of recovery in wound areas was assessed using Image J software (National Institutes of Health, Bethesda, MD, USA). The analysis was carried out in three replicates.

Colony formation assay

Cell monolayers were treated with 5FU with certain doses at the required incubation time. Following harvesting, 1x103 of 5FU treated cells were re-seeded into new 6-well plates and incubated for 9–15 days. The medium was renewed every three days during the incubation time. After incubation, the cells were fixated and stained in blue color using CellMAX Colonogenic Assay Kit (BioPioneer, USA). Colony formation rates and viability fractions of cells were calculated as described in the manufacturer's instruction. The analysis was carried out in three replicates.

Statistical analysis

The expression profiles of mRNAs and LncRNA were analyzed using an RT2 Profiler PCR Array Data Analysis (https://geneglobe.qiagen.com/tr/analyze/) with an independent t-test (SPSS). The statistical analysis of patient-derived samples including; The receiver's operating characteristic curves, and an area under the curve analysis to get the best cut-off value for each marker to discriminate the stage I/II CC tumors from the controls as well as a chi-square test to evaluate the effect of the clinicopathological data on prognosis and DFS curves of Kaplan-Meier were performed in MedCalc 12.4.0 statistical software (Ostend, Belgium).

Complementary statistics of the data in cell culture were shown as a number, percentage, mean and standard deviation. The finding of in-vitro analysis in 5FU resistance cell lineages and pcDNA3.0-PTENP1 and si-MALAT1 transfected cells were analyzed using one-way analysis of variance (ANOVA) with a post hoc test in SPSS 23 (IBM SPSS Inc, Armonk, NY) and all graphs were drawn using GraphPad Prism 6 (GraphPad Software Inc, San Diego, Calif) statistical software. The 95% confidence intervals for all values were calculated using the associated estimated standard errors. Here, $p < 0.05$ was considered significant.

Results

High expression of MALAT1 and low expression of PTENP1 foresee recurrence in Stage I/II CC

Among the analyzed 15 LncRNA, 13 LncRNAs were expressed at relatively higher and two LncRNAs were expressed at relatively lower levels in tumor tissues of stage I/II patients as compared to the negative control. Among these LncRNAs; up-regulated expression of CCAT1, HOTAIR, MALAT1, and down-regulated expression of PTENP1 in stage I/II CC tumors were in a statistically significant level (MALAT1: 5.4-fold, $p = 0.033$, CCAT1: 4.4-fold, $p = 0.039$, HOTAIR: 4.2-fold, $p = 0.041$, PTENP1: -4.8-fold, $p = 0.004$) (Fig. 1A). In addition; the expression level of MALAT1 and HOTAIR were significantly up-regulated and the expression

level of PTENP1 was significantly down-regulated in stage I/II CC tumors as compared to the positive control group; stage III/ IV tumors (HOTAIR: 2.5-fold, p = 0.02, MALAT1: 2.4-fold, p = 0.001, PTENP1: -2.2-fold, p = 0.009)

The expression level of CCAT1, HOTAIR, MALAT1, and PTENP1 were compared in tumor tissues of stage I/II patients who were recurrent (n = 21) and non-recurrent (n = 105) after 5 years follow up. According to our findings; there was no statistical association between recurrence and high expression levels of CCAT1 and HOTAIR ($p > 0.05$). The MALAT1 expression level was 2.2-fold higher and PTENP1 expression level 2.4-fold lower in recurrent tumors as compared to non-recurrent tumors (Fig. 1B; p = 0.0002, p = 0.0044, respectively). Besides, high MALAT1 expression and low PTENP1 expression led to a shorter DFS of patients than patients with their contrary expression patterns (Fig. 1C; p = 0.0009, p = 0.0158; respectively). Collectively our findings in stage I/II CC patients showed that patients whose tumors expressed a high level of MALAT1 and low level of PTENP1 are susceptible for recurrence in the following years. To validate the prognostic potential of MALAT1 and PTENP1, we generated 5-FU resistant CC cell lineages to mimic recurrent early-stage tumors.

High MALAT1 expression induce 5FU resistance

The 5FU resistant cells lineages; HT-29F1, HT-29F2, HT-29F3, HT-29F4, and HT-29FUR were generated based on the treated 5FU doses and incubation times (Fig. 2A) and they showed a 5FU resistance in an increasing level in a dose and time-dependent manner as compared to parental HT-29 cells (Fig. 2B and 2C). The cell borders of HT-29 lineages became not visible and created clusters in correlation with the increasing 5FU resistant level. Moreover, the size of clusters became bigger while the 5FU resistance was increasing. The size of clusters was the biggest in the most resistant cell lineage; HT29-FUR.

The 5-FU resistance of HT29-FUR cells was confirmed by analyzing their response to 5-FU. HT29-FUR cells were subjected to increasing concentrations of 5-FU from 0.1µg/mL to 10µg/mL. According to the findings of Annexin V analysis; HT29-FUR cells were prompt to early and late apoptosis after treatment with 1µg/mL and upper doses of 5-FU as compared to parental HT29 cells (Fig. 3; $p < 0.05$).

5FU resistance is associated with metastasis in CC

Cell migration is a hallmark of metastasis; therefore, we evaluated the invasion ability of HT-29 cells during 5FU resistance acquisition by wound healing analysis. In the HT-29FUR cells, the wound area was measured as 3.42 cm at the 0th hour, 2.48 cm at the 6th hour, 2.02 cm at the 12th hour, and 0.48 cm at the 18th hour. It was observed in these cells that the entire wound area was covered at 24 hours. Compared with HT-29 cells, the wound area decreased in HT-29FUR (Fig. 4A). After treatment with increasing doses of 5FU, the wound area was measured as 3.41 cm at 0th hour, 3.19 cm at 6th hour, 2.48 cm at 12th hour, 2.15 cm at 18th, 2.04 cm at the 24th hour. In the 18th hour, the wound area of HT-29FUR cells significantly decreased compared to HT-29 cells ($p < 0.001$) (Fig. 4B). These findings suggest that the invasive features of HT29 cells increased when they transformed to HT29-FUR.

A colony formation assay was performed to determine the aggressiveness of the cells during the 5FU resistance gain of the cells. The mean number of colonies of HT-29 cells on day 15 was determined to 29. The mean colony numbers of the sublines cells (HT-29F1-FUR) were determined as 49, 80, 120, 235, and 322, respectively. As a result of the analysis, it was observed that HT-29FUR cells formed more colonies in the 5FU environment compared to HT-29 cells ($p < 0.001$, Fig. 4C).

Epithelial cells that have undergone EMT have augmented metastasis. To analyze the metastatic potential of 5-FU resistant cell lineages, mRNA expression levels of genes that are involved in the EMT process were investigated in HT-29, HT-29F1, HT-29F2, HT-29F3 HT-29F4, and HT29-FUR, comparatively. The mRNA expression levels of *CDH2*, *Slug, Zeb1*, *Twist*, and *Vimentin* were upregulated in 5FU resistance sublines cells compared with HT-29; however, these results were not significant ($p > 0.05$). The expression of *E-CADHERIN* level was significantly downregulated, the expression levels of *SNAIL* were significantly upregulated in HT-29F1-FUR compared with HT-29. In addition, we found that the expression of MMP-9 was significantly higher in only HT-29FUR compared to HT-29 (3.4-fold, $p = 0.014$).

MALAT1 play role in 5FU resistance in CC

To determine whether MALAT1 and PTENP1 contribute to the development of 5FU-resistance in colon cells, real-time PCR was explored in the HT-29FUR and HT-29 cells. As showed in Fig. 5A, expression of MALAT1 was upregulated in 5FU-resistant sublines from HT-29F3 compared with HT-29 cells ($p < 0.05$). In addition, the expression PTENP1 was downregulated in HT-9FUR compared with HT-29 cells. After transfection of pcDNA-PTENP1 and si-MALAT1, PTENP1 expression increased in pcDNA-PTENP1 transfected cells, while MALAT1 expression was decreased in si-MALAT1transfected cells as compared to non-transfected HT-29 FUR cells. (Fig. 5B). The cells became sensitive to 5FU after si-MALAT1 transfection, with only 38.8% of viable cells remaining after exposure to 2 µg/mL 5FU for 72 hours (Fig. 5C).

To determine if knockdown of MALAT1 affects CC migration and colony formation, we performed wound-healing and colony formation assay (Fig. 5D). The migration and colony formation capacity of the CC cells were markedly decreased when MALAT1 was blocked by siRNA. These data demonstrate that endogenous activation of MALAT1 promoted CC cell proliferation and invasion.

The mRNA expression of 88 genes that are involved in signaling pathways affected by colorectal carcinogenesis was investigated in pcDNA-PTENP1 and si-MALAT1 expressed cells as compared to non-transfected HT-29 cells. According to the findings, the mRNA expression level of CDH1 was increased (7.21-fold, $P < 0.001$) while *CD133* (4.44-fold, $P = 0.0021$) and *Mitogen-Activated Protein Kinase Kinase Kinase 1* (*MAP3K1*) (5.56-fold, $P < 0.0001$) were decreased in si-MALAT1 expressed cells as compared to HT-29 cells. mRNA expression patterns of those of genes that were not affected by pcDNA-PTENP1 expression (Fig. 5E). Collectively, these findings suggest that an attenuated expression of MALAT1 in 5FU resistant cells reduces metastasis susceptibility by reversing EMT processes.

Discussion

CC is one of the most common malignant diseases in humans and the third leading cause of cancer-related deaths worldwide.[1] Although recent advances in diagnostics and therapeutics have improved the clinical outcomes for Stage I/II CC, a significant proportion of these early-stage tumors still experience systemic recurrence, also known as distant metastasis. There are few reliable markers available to accurately predict liver metastasis in Stage I/II CC, and CC cells are prone to drug resistance, leading to the failure of chemotherapy. Thus, effective treatment and prevention of metastasis and recurrence remain a challenge [17,18]. Furthermore, the need for adjuvant chemotherapy is still controversial in stage I/II CC [19]. According to clinical guidelines, a mucinous component and perineural invasion are poor prognostic factors in stage I/II CC, and adjuvant chemotherapy is recommended for patients with these "high-risk" features. However, a poor prognosis may be observed in patients who do not carry these features [20]. In the present study, first, we determined the effect of the dysregulation of LncRNAs on recurrence in stage I/II CC tumors that did not have poor prognostic features. We evaluated 15 metastasis-associated LncRNAs and we found that PTENP1 and MALAT1 expression were dysregulated in recurrent-Stage I/II CC.

PTENP1 is the pseudogene of the PTEN, which is a tumor suppressor LncRNA [21]. An increasing number of studies have shown that PTENP1 functions as a competing endogenous RNA to suppress tumor progression [21, 22]. According to Gharib E et, al. decreased expression of PTENP1 could be an early detection marker for colorectal tumors [23]. On the other hand, the formation of colorectal tumors may be different depending on their origin is colon or rectum. Therefore, in this study, we confirmed the role of PTENP1 in tumorigenesis a more homogenous cohort that consists of only stage I/II tumors localized only in the colon. Similar to Gharib et al, we defined the decreased expression of PTENP1 in colon tumors. Studies indicate that 5FU resistant colon cells are more susceptible to promote metastasis. Therefore, to confirm the marker potential of decreased expression of PTENP1 to predict metastasis, PTENP1 expression was analyzed in 5FU resistant HT29 cell lineages. The findings indicated that the PTENP1 expression was negatively correlated with the increasing level of 5FU resistance and when PTENP1 expression was replaced in the 5FU resistant cells, 5FU response was increased. However, the replacement of PTENP1 did not affect the metastasis susceptibility in 5FU resistant cells. These findings suggest that, although downregulation of PTENP1 may increase the metastasis potential, it appears as not a direct factor to lead metastasis. Instead, it accelerates the metastasis processes due to it leads to 5FU resistance.

MALAT1, also known as nuclear-enriched transcript 2 (NEAT2), is a prognostic parameter for survival and associated with poor prognosis after surgical treatment in solid tumors [24–28]. Studies reported the enhanced expression pattern of MALAT1 in recurrent colorectal cancer and metastatic tumors as compared to non-recurrent primary tumors [25,29]. In addition, Li P et al. demonstrated a negative correlation of the high MALAT1 expression and chemotherapy response in-vitro and in a patient cohort [29]. On the other hand, our understanding of the predictive potential of high MALAT1 expression for liver metastasis in the early stages of colon tumors and its function during the process of 5FU-based chemotherapy failure is remaining insufficient [30–32]. In our stage I/II patient-derived tumor cohort we

defined the high expression of MALAT1 which relapsed in the following 5 years. In addition, the MALAT1 was expressed increasingly in HT-29 cell lineages depend on the level of 5FU resistance. Moreover, when MALAT1 expression attenuated in HT29-FUR cells, which is the most resistant HT-29 cell lineage to 5FU, while the sensitivity of 5FU increased, the features of metastasis such as, colony formation, and wound healing, were decreased. In addition, suppression of MALAT1 resulted in stimulation of *CDH1* expression and suppression of *MAP3K1* and *CD133* expressions which indicates a decelerate of EMT processes as well as the transformations to the metastatic features in 5FU resistant cells. These findings imply that 5FU therapy could not be beneficial for patients with high MALAT1 expressed tumors. Meanwhile, these findings also promise that high MALAT1 expression as an early event has the potential to foresee a future recurrence risk in the time of diagnosis.

In conclusion, in this study, a homogeneous early-stage CC patient cohort was under investigation. However, validating the expression pattern of PTENP1 and MALAT1 together with clinicopathological characteristics in larger study cohorts will clarify how these LncRNAs involve in the molecular pathways of colon tumorigenesis and 5FU resistance. In addition, the reflection of these LncRNA expression patterns on the functions of targeting proteins is an open area to investigate. Based on the current study, our findings suggest that a low expression of PTENP1 and a high expression of MALAT1 in stage I/II colon tumors could predict metastasis due to the insufficient 5FU response. However, it should be noted that although high MALAT1 expression heralds a future metastasis because it also promotes 5FU resistance, 5FU mediated chemotherapies may not be advised, instead, a MALAT-1 targeted cell-based therapy will be more beneficial for these patients.

Declarations

Compliance with Ethical Standards:

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (2014-12/16).

Author contributions

Conceptualization: Secil Ak Aksoy, Berrin Tunca; Methodology: Secil Ak Aksoy, Berrin Tunca, Ersin Ozturk, Formal analysis and investigation: Secil Ak Aksoy, Nesrin Ugras, Writing - original draft preparation: Secil Ak Aksoy, Yuncay Yılmazlar, Gulsah Cecener Writing - review and editing: [full name], Berrin Tunca, Unal Egeli, Omer Yerci, Gulcin Tezcan

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No conflict of interest exists.

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Figures

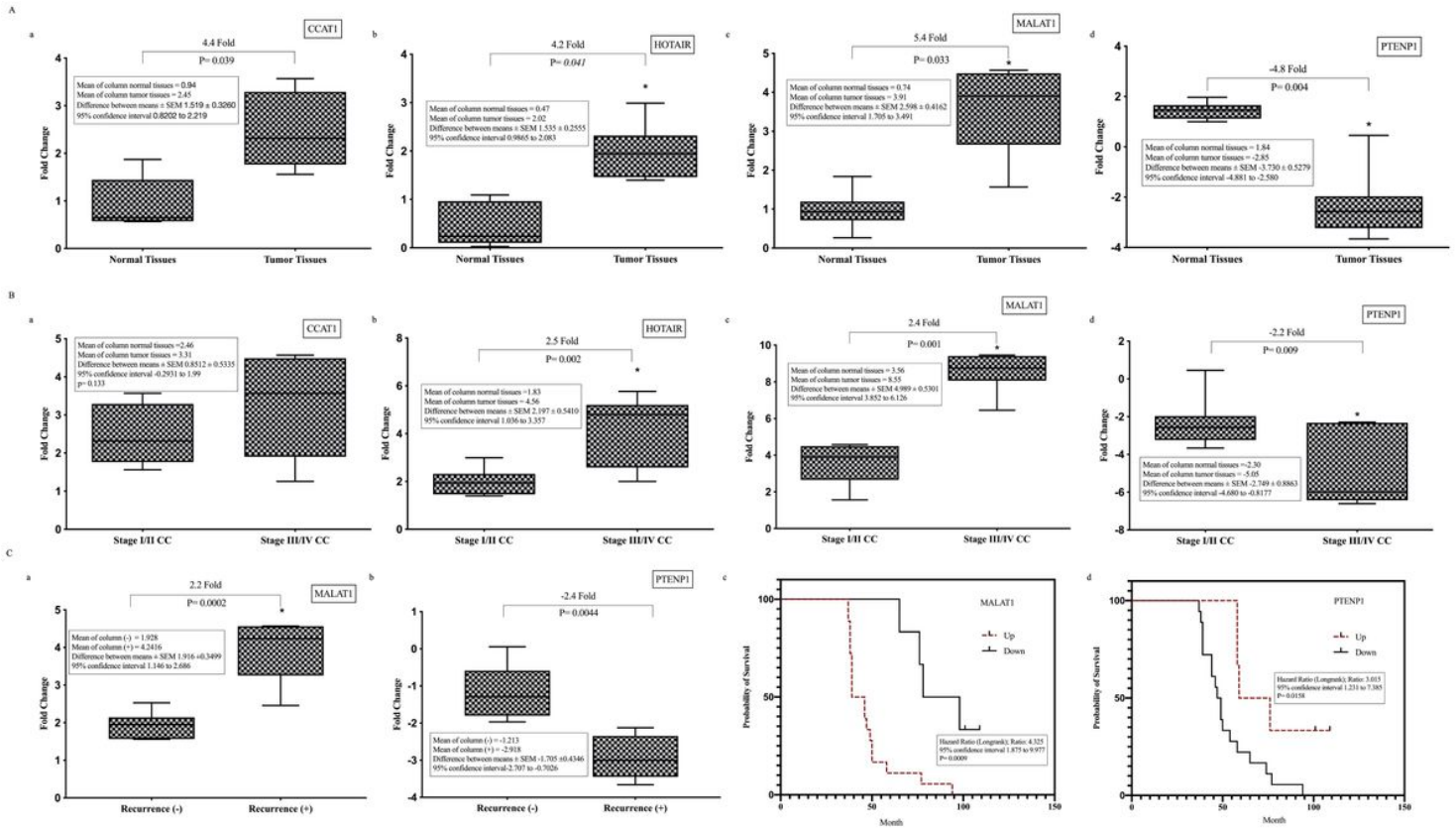

Figure 1

(A) The expression profiles of CCAT1 (a), HOTAR (b), MALAT1 (c) and PTENP1 (d) in CC compared with normal colon tissues. (B) The expression profiles of CCAT1 (a), HOTAR (b), MALAT1 (c) and PTENP1 (d) in Stage I/II compared with Stage III/IV CC. (C). The expression profiles of MALAT1 (a) and PTENP1 (b) in recurrent Stage II CC. The Kaplan-Meier curves of MALAT1 (c) and PTENP1 (d).

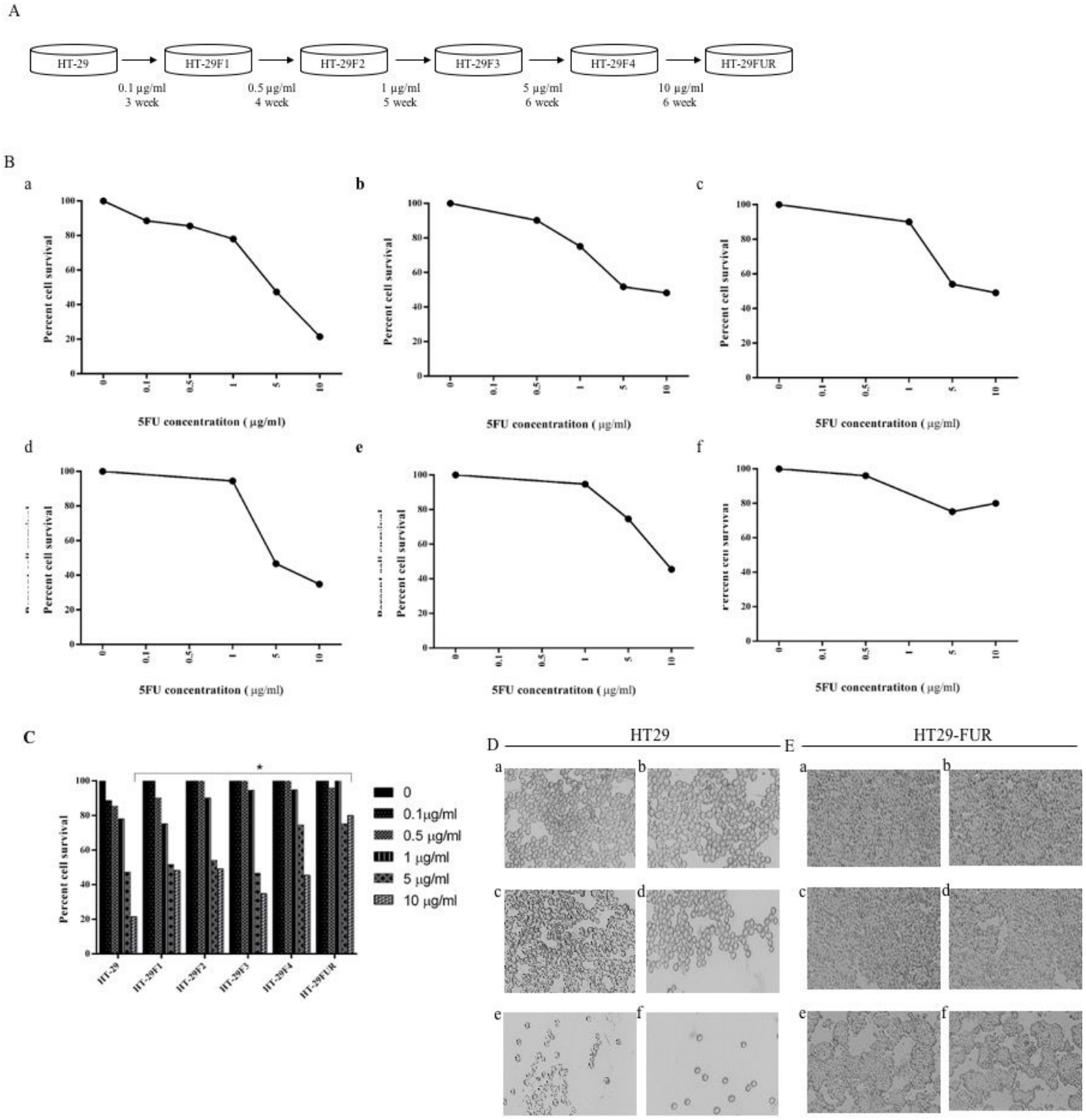

Figure 2

(A) A histogram of acquired drug resistance to 5-FU over 6 months. (B) Cell viability of (a) HT-29, (b) HT-29F1, (c) HT-29F2, (d) HT-29F3, (e) HT-29F4 and (f) HT-29FUR cells was measured by a WST-1 assay. (C) The proliferative ability of resistant cell-sublines were less affected by 5-FU compared to HT-29 parental (*p < 0.05). (D) Inverted microscopy images of HT-29 and (E) HT-29FUR cells after (a) 0 µg/ml, (b) 0.1 µg/ml, (c) 0.5 µg/ml, (d) 1 µg/ml, (e) 5 µg/ml and (f) 10 µg/ml.

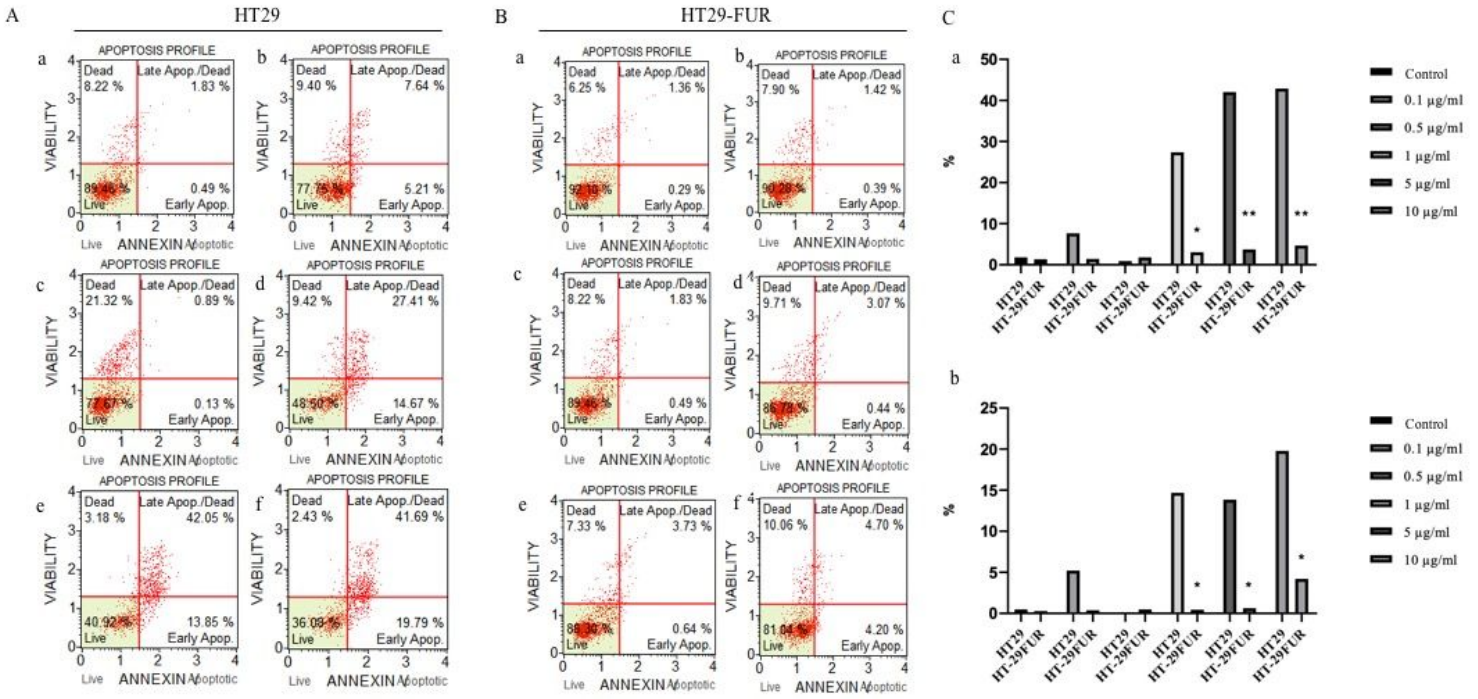

Figure 3

The viability, necrosis, late apoptosis and early apoptosis rates in HT-29 (A) and HT-29FUR (B) cells treated with (a) 0 µg/ml, (b) 0.1 µg/ml, (c) 0.5 µg/ml, (d) 1 µg/ml, (e) 5 µg/ml and (f) 10 µg/ml 5FU for 72 hours. (C) A statistically significant increase was found in early and late apoptosis percentages in HT-29FUR cells compared to HT-29 cells (a) Early apoptosis and (b) late apoptosis rates in HT-29FUR cells compared to HT-29 cells after 72 hours of 5FU treatments at various doses (*p <0.05, **p <0.001). Results are representative of three independent experiments.

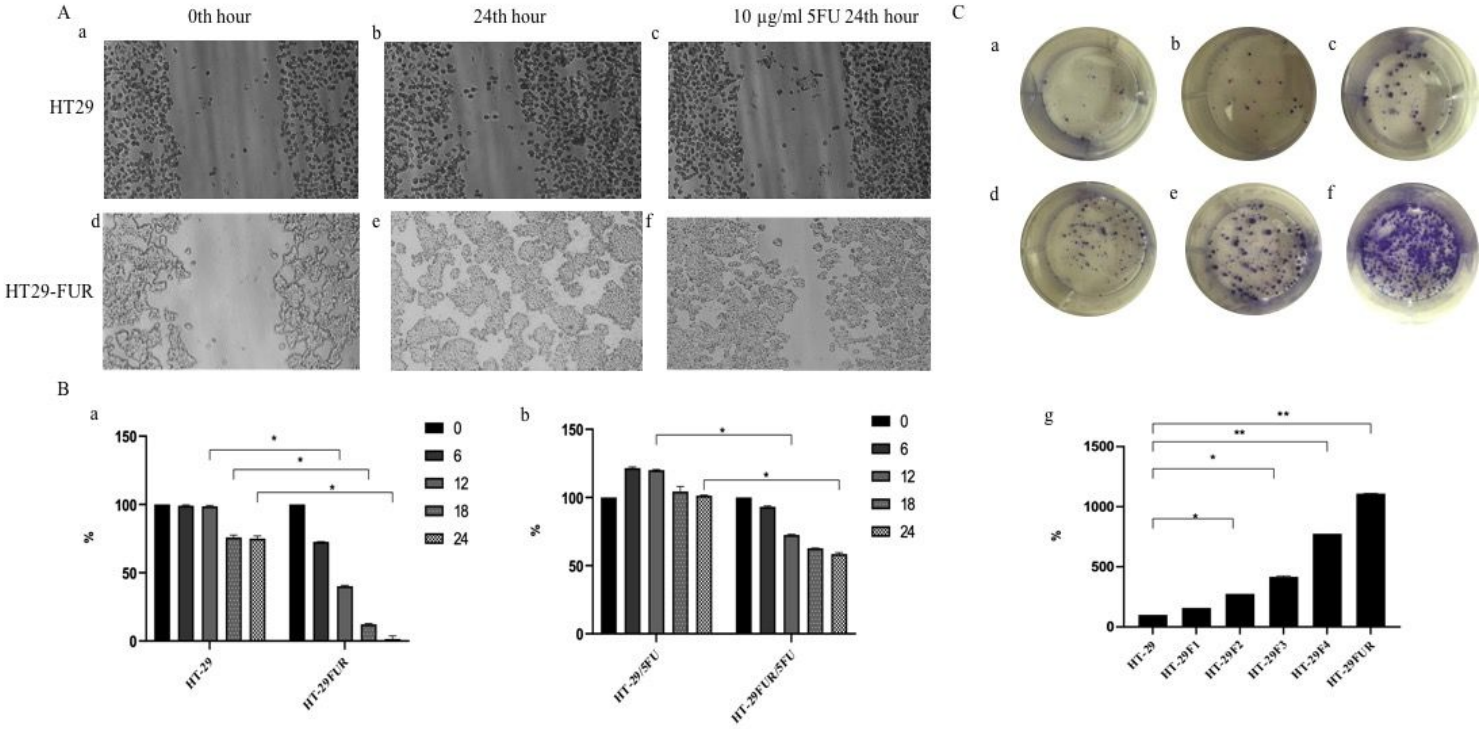

Figure 4

(A) Images of wound area of (a) HT-29 at 0th, (b) HT-29 at 24th, (c) HT-29 after treatment with 10 µg/ml 5-FU at 24th (d) HT-29FUR cells at 0th (e) HT-29FUR cells at 24th

(f) HT-29FUR after treatment with 10 µg/ml 5-FU at 24th (B) In HT-29FUR cell-subline, the size of wound area recovered significantly faster than HT-29 cells (a) similar to untreated HT-29FUR cell-subline, in 24th hour, the wound area of HT-29FUR cells significantly decreased compared to HT-29 cells (b). (C) Colony-forming capabilities of (a) HT-29, (b) HT-29F1, (c) HT-29F2, (d) HT-29F3, (e) HT-29F4 and (f) HT-29FUR. HT-29FUR cells formed more colonies in 5-FU environment compared to HT-29 cells (g).

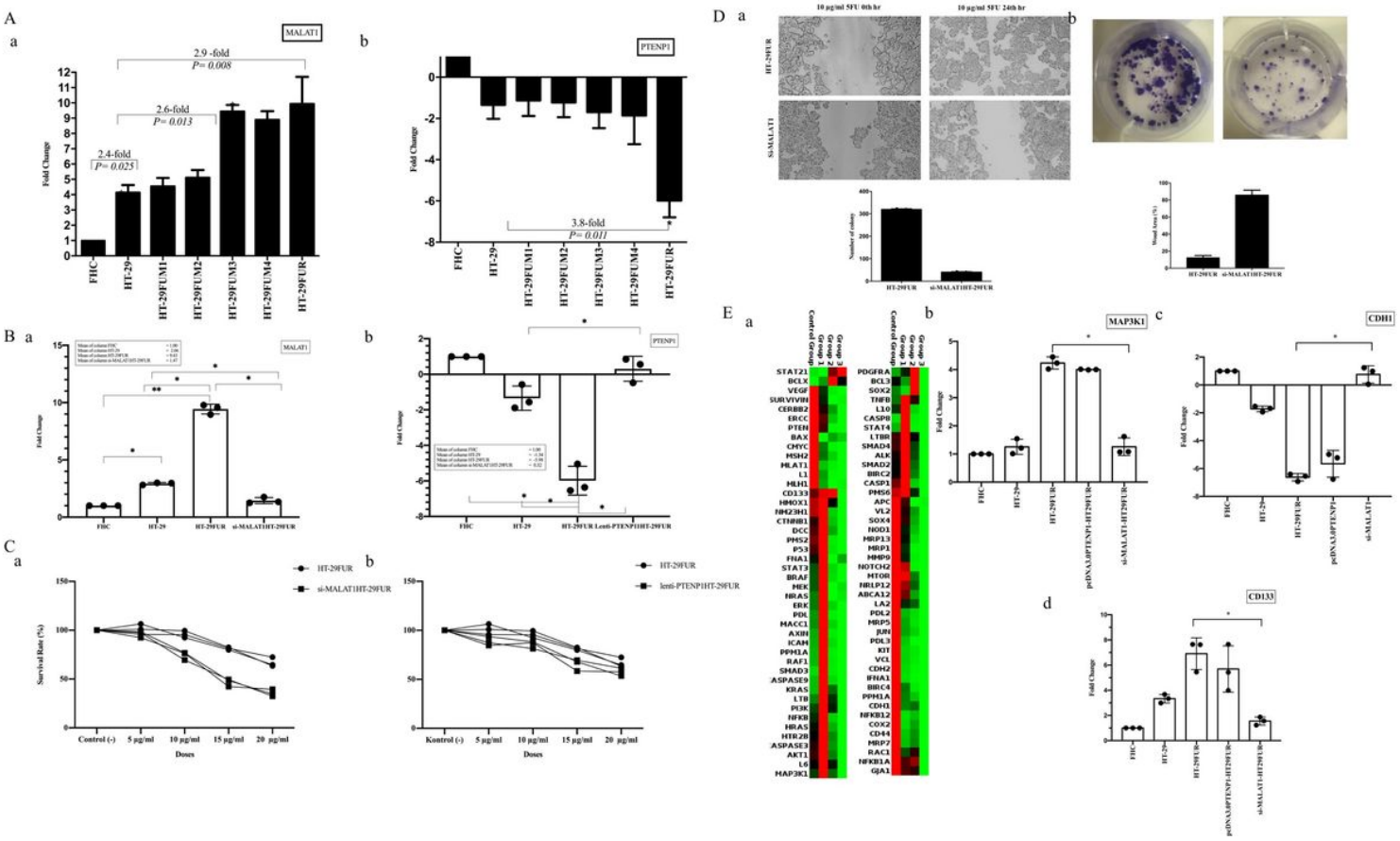

Figure 5

(A) The expression profiles of PTENP1 (a) and MALAT1 (b) in FHC, HT29, HT-29F1, HT-29F2, HT-29F3, HT-29F4 and HT-29FUR cells. (B) The expression profiles of PTENP1 (a) and MALAT1 (b) and both (c) in transfected-cells. (C) Cell proliferation after 5FU exposure in control and transfected cells .Cell proliferation in si-MALAT1HT-29FUR cells after 5FU exposure (a) cell proliferation in Lenti-PTENP1HT-29FUR cells after 5FU exposure(b). (D) Wound closure in si-MALAT1HT-29FUR cells compared to HT-29FUR cells (a) and Colony formation ability (b) (F) Gene expression differences between cell groups CDH1 (b), MAP3K1 (c), CD133 (d).*Control: HT-29, Group 1: HT-29FUR, Group 2: pcDNA-PTENP1, Group 3: si-MALAT1