Securin overexpression correlates with the activated Rb/E2F1 pathway and Histone H3 epigenetic modifications in raw areca-nut induced carcinogenesis in mice

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Abstract

Background

Raw areca-nut (RAN) consumption induces oral, esophageal and gastric cancers which are significantly associated with the overexpression of pituitary tumor transforming gene1/Securin and chromosomal instability (CIN). Since the molecular mechanism underlying Securin upregulation remains unclear, this study is intended to investigate the association of Securin upregulation with Rb-E2F1 circuit and epigenetic histone (H3) modifications pattern both globally and in the promoter region of Securin gene.

Methods

Six groups of mice were used and in the treated group each mouse consumed 1 mg of RAN-extract with lime per day *ad libitum* in the drinking water for 60 days after which the dose was increased by 1 mg after every 60 days. Histopathological evaluation of stomach tissues was done and Securin expression was analysed by immunoblotting as well as by immunohistochemistry. ChIP-qPCR assay was performed for evaluating the recruitment of different histone modifications in the two regions of Securin promoter.

Results

All mice developed gastric cancer with Securin overexpression after 300 days of feeding. Immunohistochemistry data revealed hyperphosphorylation of Rb and upregulation of E2F1 in the RAN-treated samples. Increased trimethylation of H3 Lysine4 and acetylation of H3 Lysine9 and 18 both globally and in the promoter region of Securin gene was observed by increasing the level of lysine-N-methyltransferase2A, lysine-acetyltransferase, EP-300 and PCAF after RAN-treatment. ChIP-qPCR data show an increased recruitment of H3K4me3, H3K9ac and H3K18ac in the promoter of Securin gene after RAN-exposure

Conclusions

In light of the present results, it seems that RAN-mediated pRb-inactivation induced Securin upregulation, a putative E2F1 target, by inducing misregulation in chromatin remodeling in its promoter region which led to transcriptional activation and subsequently develop chromosomal instability. Therefore, the present results have led to the hypothesis that RAN-induced changes in the epigenetic landscape, Securin overexpression and subsequent elevation of chromosomal instability are probably a by-product of the inactivation of the pRb pathway.

Introduction

Mammalian securin which has been isolated initially from rat pituitary tumor cells as pituitary tumor transforming gene 1 (PTTG1) [1], is an oncogene and has been implicated in the development and progression of several malignancies [2]. It encodes a protein which prevents separin from promoting sister chromatid separation during mitosis [3]. This gene product is involved in cell cycle progression, p53-mediated apoptosis, transcription activator of several other oncogenes [4] and DNA-repair [5]. It has been demonstrated that higher expression of Securin, induction of precocious anaphase (premature separation of sister-chromatids) and chromosomal instability have been associated with an increased risk of raw areca-nut (RAN) induced oral, esophageal and gastric cancers in both human and mouse [6, 7]. In fact, it has been proposed that these parameters can be considered as screening markers for identification of mitotic checkpoint defects during early days of RAN exposure. Traditionally people of Northeastern region of India consume betel-quid consists of RAN, lime and a small portion of betel-leaf without tobacco. After chewing, they usually swallow the whole quid which is responsible for not only an induction of oral cancer but also for esophageal and gastric cancers. Higher DNA damage, p53 overexpression, greater delay in cell kinetics and lower GSH-level in peripheral blood lymphocytes have been demonstrated in heavy RAN-chewers than non-chewers [8]. Such observations prompted to propose that besides cytogenetical parameters, the level of endogenous GSH and p53 protein could act as an effective biomarker for RAN chewers [8]. The mutagenicity and genotoxicity of RAN-alkaloids has been demonstrated in several short-term assays and it has been suggested that RAN should be considered as a human carcinogen since it induces preneoplastic as well as neoplastic lesions in experimental animals [9].

Upregulation of Securin and subsequent dysregulation of chromosome segregation leading to chromosomal instability have been observed not only in human cancer cell lines but also in a vast array of malignancies including pituitary, colorectal, thyroid, lung, prostate, oral and esophageal squamous cell carcinoma [10–18]. Increased level of Securin is correlated with higher tumor grade, invasiveness and tumor vascularity [19]. Thus, it has been proposed that Securin level may be considered as a molecular marker which can be a potential therapeutic target for many cancers [20]. However, it is interesting to note that despite its clinical relevance, the molecular mechanisms underlying Securin abundance remain elusive.

It has been observed that hPPTG1 expression was reduced in bladder cancer cells after knockdown of E2F3 by measuring the expression of cDNA microarray analysis [21]. Further, it was shown that hPTTG1 may act as a direct E2F1 target and both were concordantly overexpressed in $Rb^{+/-}$ murine pituitary tissues and also in human pituitary tumors [22] Therefore, the present study is intended to evaluate the expression of E2F1 and Rb-phosphorylation and their association with the Securin overexpression. In addition, epigenetic histone modifications pattern in the promoter region of Securin gene will also be evaluated because such modifications do have profound effects on gene promoter activity [23, 24]. Chromatin remodelling occurs through the posttranslational modifications of basic amino acid residues of histone tails, which either causes activation or repression of gene expression [25–27]. It is now well documented that the structure and integrity of the genome can be altered by disrupting this complex

epigenetic control mechanism which ultimately alter the expression of genes that critically involved in tumorigenesis [26, 28].

It has become increasingly clear that both environmental factors and the lifestyle can promote a wide range of epigenetic modifications that are causally involved in cancer development and progression [29]. Therefore, the objective of this study is to analyse the status of RAN-induced E2F1 expression, pRb-phosphorylation and posttranslational histone H3 modifications both at the global level and in the promoter region of Securin gene by immunohistochemistry and standard chromatin immuno-precipitation-qPCR (ChIP-qPCR) protocol. Our data reveal that Rb-inactivation releases E2F1 to induce PTTG1/Securin expression and also show its ability in exerting broader effects on transcriptional control and chromatin structure through epigenetic histone modifications in RAN-induced gastric cancers.

Materials And Methods

Preparation of extracts

RAN were ground into fine powder and 100 g of the powder were extracted with 125 ml of distilled water and mixed thoroughly to give a smooth paste for preparation of an aqueous extract of RAN. After 24 h at 4^{o}C, the paste was stirred for 3 h at room temperature and the aqueous extract was collected by centrifugation. This extraction procedure was repeated once more by adding 125 ml of water to the residue. Both extracts were pooled, representing 100 g of RAN in 250 ml distilled water, filtered and frozen at -80°C. The filtrate was lyophilized in a Scanlaf Coolsafe Lyophilizer (Lynge, Denmark). The lyophilized mass was kept at 4°C until use. The extract contained 0.9 g/100 g water-extractable material.

Animals maintenance and treatment

Swiss albino mice (25–30 gm) and aged 2–3 months were maintained in the laboratory in community cages and housed in the Animal Resource Facility of the university under the following conditions: 12-h dark/12-h light cycle, 20±2°C temperatures and 50±10% humidity. Standard mouse diet (NMC Oil Mills Ltd., Pune, India) and water *ad libitum* were used in all experiments. A total of six groups of mice (n=7 in each) were used 0, 60, 100, 180, 240 and 300 days for different experimental analysis. One group was treated with simple drinking water considered to be untreated control whereas other five groups were administered RAN extract *ad libitum* in the drinking water with slaked lime (calcium hydroxide; pH 9.8). Each mouse consumed 1 mg of extract per day. Such oral administeration was continued for 60 days after which the dose was increased from 1 mg to 2 mg per day till 120 days. This way every 60 days after, the dose was increased by 1 mg per day consumption. In the present study, the mice were fed till 300 days. The dose and the treatment pattern were similar to our earlier study where it was shown that continuous *ad libitum* administration of RAN extract with lime in drinking water for 220 days or more can induce stomach and esophageal cancer in mice [6].

Histopathological evaluation

Stomach tissues of mice were collected from untreated and treated with RAN + lime for 300 days and preserved in 10% formalin. Three mice were selected from the untreated group and none of them showed any indication of tumor externally. All the seven mice in the treated group for 300 days were selected for histological evaluation. Tissues were processed for histological sectioning as per standard protocol [30]. Formalin-fixed paraffin embedded tissue blocks were serially sectioned (5 µm) with a microtome (Leica Biosystems, Wetzlar, Germany) and stained with hematoxylin and eosin [31]. Sections were then observed under a light microscope and photographed (Carl Zeiss, Oberkochen, Germany).

Immunoblotting

Cells were collected from the inner layer of stomach from untreated (n=2) and RAN+lime treated mice for 300 days (n=3). The cells were washed with ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) and total protein was extracted with a lysis buffer containing 0.1% SDS, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 50 mM sodium fluoride, 100 U/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. After centrifugation, the cell lysate was collected and the protein concentration was determined using the bicinchoninic acid protein assay. Equal amount of protein (40 µg/well) was subjected to Novex Tris-Glycine 4–20% gradient gels and electrophoresis was performed in NuPAGE electrophoresis system (Invitrogen, California, USA). Then the proteins were transferred to a polyvinylidene difluoride membrane (Sigma) and probed with 1:1000 dilution of a mouse monoclonal antibody against Securin (DCS-280; ab3305; Abcam, California, USA) and β-actin (AC-15; ab6276; Abcam, USA). Alkaline–phosphatase conjugated anti-mouse IgG (Abcam, USA) used as secondary antibodies and immunodetection was performed by treating the blot with the substrate solution of BCIP/NBT (Bangalore Genei, India).

Immunohistochemistry (IHC) analysis

Stomach tissues of mice were collected from untreated and treated with RAN + lime for all the different time periods and preserved in 10% formalin. Four mice were selected from each group. Tissues samples were dehydrated, paraffin embedded and sectioned with a microtome. Briefly, after blocking for endogenous peroxidase activity, the sections of stomach tissues were incubated with anti-Securin (DCS-280; ab3305; Abcam, UK), anti-H3K4me3 (Histone H3 Lysine 4 trimethylation) primary antibody (ab8580; Abcam, UK), anti-H3K9me3 (Histone H3 Lysine 9 trimethylation) primary antibody (ab8898; Abcam, UK), anti-H3K9Ac (Histone H3 Lysine 9 acetylation) primary antibody (ab12179; Abcam, UK), anti-H3K18ac (H3 Lysine18 acetylation) primary antibody (ab1191; Abcam, USA), anti-Rb-phosphorylation primary antibody (SC-271930; Santa Cruz Biotechnology, USA) and anti-E2F1 primary antibody (SC-22820; Santa Cruz Biotechnology, USA). IHC analysis was performed with a Strept-Avidin Biotin Kit (Dako, Agilent Technologies Company, Denmark). The scoring of immunohisto-chemical stains in each specimen was determined using a histological score (H) [32] (please see Supplemental Information). Only Histone 3 antibody (ab1791; Abcam, UK) was used as an internal control.

Chromatin immunoprecipitation assay (ChIP)

ChIP assay for detection of posttranslational histone modifications patterns in the promoter region of Securin gene were performed in mouse stomach cells. Stomach epithelial cells from four different animals at each point were lysed, sonicated and incubated with antibodies specific to H3K4me3 (ab8580, AbCam, UK), H3K9Ac (ab12179), H3K9me3 (ab8898), H3K18Ac (ab1191) and Histone 3 (ab1791) with protein A/G beads (Pierce™ Protein A/G Agarose, Cat no. 20421) incubated for overnight at 4°C. The methodology of ChIP in details is mentioned in the Supplementary section.

Quantitative PCR (qPCR; BioRad CFX system) was used to quantitate amounts of DNA fragments in the immunoprecipitated samples from the ChIP analyses. qPCR was performed with the reagents containing SYBR green and specific two primer sets located within -478bp to -874bp of promoter of PTTG1 of mouse confirmed by sequencing (Science genome browser) of the qPCR products. Samples were heated to 95°C for 5 min and then amplified for 45 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Immunoprecipitated DNA was detected by qPCR and normalized with input DNA. Enrichment was calculated relative to input. qPCR products were purified using SIGMA PCR clean up kit (NA 1020) and sent for sequencing to Agrigenome, Kochi, India.

RNA extraction and qRT-PCR

qRT-PCR was performed to assess the transcriptional levels of KMT2A (lysine methyltransferase2A), KAT2A (lysine acetyltransferase2A), EP300 (lysine acetyltransferase KAT3B), P300/CBP-associated factor (PCAF) also known as K(lysine) acetyltransferase 2B (KAT2B), HDAC3 (histone deacetylase 3), KDM4C (histone demethylase) and the reference gene *GAPDH*. Cell lysates were collected and the RNA was extracted and purified using the Rneasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of the RNA into cDNA was then performed using QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). qRT-PCR was performed in a Bio-Rad CFX96 Real-Time PCR Detection System using SYBR Green PCR Master Mix (Life Technologies, Delhi, India). The primers used for qRT-PCR were listed in Supplementary Table S1.

Statistical analysis

For comparing the expression Securin between untreated and different duration of treated groups unpaired Student's t-test was performed for statistical analysis. The statistical significance in the level of Histone3 K4-trimethylation, K9-acetylation, K9-trimethylation, K18-acetylation, pRb-phosphorylation, E2F1 activities and the expression of several epigenetic chromatin modification enzymes between treated and untreated groups, was determined by one-way ANOVA. The Turkey test was used for post hoc analysis. The results are shown as means ± SEM and $P < 0.05$ is considered as statistically significant. Statistical analysis was performed using GraphPad Prism 5.0.

Results

General observations

In total 42 mice, distributed in six groups, were used at different time points for different experimental analysis. After 300 days of feeding RAN extract with lime, all the seven mice developed stomach cancer. The histological section clearly differentiated between normal and tumorous stomach (Fig. 1A a-f) in mice.

Securin over-expression through immunohistochemical staining

The expression of Securin was evaluated by immunostaining in a panel of mouse stomach samples collected from untreated and treated with RAN+lime for 60, 100, 180, 240 and 300 days (Fig. 1B). The expression of Securin was increased gradually from 60 to 300 days of feeding with RAN+lime than untreated control. At each point, five mice were used. The H-score in untreated control showed less than 40 and it reached to around 200 after 300 days of feeding (Fig. 1C).

Over-expression of Securin gene through immunoblotting

The level of Securin protein in stomach cells from mice after administration of RAN extract with lime for 0 and 300 days of feeding was examined by immunoblotting. Results indicate that the expression of Securin gene was elevated significantly after 300 days of feeding (Fig. 1D and 1E).

Global methylation and acetylation pattern of histone H3 lysine 4, 9 and 18 in stomach cells

To examine the pattern of tri-methylated and acetylated histone H3 at lysine 4, 9 and 18, we conducted immunohistochemistry on serial sections of mouse stomach (Fig. 2). The histo-chemical staining of H3K4me3, H3K9ac, H3K9me3 and H3K18ac appeared as brown particles and localized primarily within nuclei of stomach epithelial cells. As shown in Figure 2A to 2F, the staining pattern of H3K4me3, H3K9ac and H3K18ac was very weak in untreated stomach samples which was gradually stronger in 100 and 300 days treated samples. The mean H-score of H3K4me3, H3K9ac and H3K18ac was increased significantly after 100 and 300 days of RAN-treatment with respect to untreated control. Interestingly, the staining pattern of H3K9me3 was reduced significantly after 100 and 300 days of RAN-treatment with respect to untreated control (Fig. 2 G and H).

The IHC images with only Histone 3 antibody in stomach cells are shown similar in untreated and treated samples (Fig. S1 in the supplementary section).

Hyperphosphorylation of Rb and increased E2F1 expression after RAN-treatment

Immunohistochemical assays of phosphor-retinoblastoma (pRb) and E2F1 expression in mouse stomach cells with and without RAN-treatment were demonstrated in Fig. 3A-3D. Hyperphosphorylation of RB and consecutive stimulation of E2F1 was clear in the RAN-treated samples.

Securin gene structure in mice and primer design

The entire mouse PTTG1/Securin gene spans about 7 Kb and is composed of five exons and four introns. The exons are sized about 391, 179, 94, 150, and 131 bp, respectively *and four introns are about 0.9, 2.1,*

2.0 and 0.8 Kb, respectively (Fig. 3E) [33].The translation start-site (ATG) is located 303 bp downstream of the single transcription start site and from deletion scanning study of an active 4.3 kb upstream region *revealed that* sequences −313 to −150 bp to be critical for promoter activity. The Securin gene promoter-specific two primers were designed from UCSC browser and with the help of Primer 3 software (Version 0.4.0) within the region from -478 to -874 which is further upstream in the promoter region. Usually, the core enhancer sequence is present in the upstream region of the Securin gene promoter in both rat and human, which is critical for Securin transcriptional activation [34,35].

Primer details are given below:

Primer 1: Forward 5'-GGCCTGTTCCCCTAGAGATT-3'

Reverse 5'- CAGGCTTTTCGGAAACTCAC-3'

Primer 2: Forward 5'-CAGGCCATCCTGGTCTACAT-3'

Reverse 5'-GGAGAGATTCCTGGGCAGTT-3'

ChIP analysis of chromatin composition

In order to determine the recruitment of posttranslational modifications of histone H3 (methylation/ acetylation of histone H3 lysine 4, 9 and 18) in the promoter region of Securin gene, chromatin immunoprecipitation (ChIP) assay was performed on stomach tissues in untreated, 100 and 300 days of RAN+lime treated samples. The assays were then analyzed by qPCR with two primers targeting the promoter of Securin gene. Fig. 4 shows the results of ChIP experiments (enrichment of histone marks on Securin promoter indicated in % input). The data show that RAN+lime treatment led to an increase in the levels of H3K4Me3, H3K9ac and H3K18ac significantly in 300 days treated samples (Fig. 4 A - 4 F). Interestingly, the level of H3K9me3 was reduced significantly in 100 and 300 days treated samples compared to untreated control (Fig. 4 G and H). The results indicate that the amount of DNA fragments retrieved from the immunoprecipitated samples was more in the -478 to -574 region of the promoter for H3K4Me3, H3K18ac and H3K9me3 whereas for H3K9ac the region was further upstream of the promoter (-785 to -874).

The product size of the qPCR was 107 bp for primer 1 and 90bp for primer 2. Both the products were sequenced and then blast (NCBI nucleotide blast) with the mouse genomic sequences. It was matched with the mouse PTTG1/Securin gene present at chromosome 11. Both the sequenced portion were present within the promoter region of Securin gene and precisely it was located in -478 to -584 bp for primer 1 and -785 to -874 for primer 2 (Fig 5).

Gene expression profiles of histone modifying enzymes after RAN-treatment

We have shown that RAN-exposure increases the expression of Securin in stomach cells by increasing H3K4me3 and both H3K9 and H3K18 acetylation in the promoter region of Securin. The present qRT-PCR

data show that lysine methyltransferase2A (KMT2A), lysine acetyltransferase2A (KAT2A), lysine acetyltransferase3B (EP300) and P300/CBP-associated factor (PCAF) are increased significantly after 300 days of feeding with RAN+lime (Fig. 6 A and B). The expression pattern of a histone demethylase (KDM4C), specifically targets tri- and di-methylated lysine 9 of histone H3 (H3-K9me3 and me2), was analysed and significant upregulation was observed after 300 days of feeding (Fig. 6C). Consistent with the KAT2A and KAT3B upregulation, the expression of histone deacetylase3 (HDAC) is downregulated (Fig. 6D). We have shown that RAN exposure alters the gene expression pattern of chromatin modification enzymes, thereby affecting the dynamics of histone modifications that regulate transcriptional control *in vivo* in the mouse stomach cells.

Discussion

It is known that increased level of Securin facilitates genome instability which is associated to a subset of genomic rearrangements leading to a worse prognosis in a broad range of cancerous tissues [17, 18]. Extensive research on this gene has been performed due to its clinical importance, however, the precise mechanisms by which Securin induces its oncogenic function and the mechanisms that regulate its overexpression remain unknown. This study was carried out in mice because its exposure to RAN was regulated in a proper manner which was not possible in human and moreover interference of several other compounding factors (tobacco chewing or smoking, alcohol consumption and various types of non-vegeterian foods) lead confusion. Here, the mice were treated with RAN extract with lime whose dose was increased periodically in order to mimic the human consumption style where the dose is increased gradually. Studies in different animal species have shown tumour induction in esophagus and stomach tissues by RAN extract which was administered by different means such as oral intubation [36], mixed with the diet [37] or drinking water [6]. Here, mouse stomach was considered because of its greater exposure after *ad libitum* admisteration of RAN-extract with lime in drinking water.

Sequencing scan in sixteen human pituitary adenoma biopsies failed to identify any promoter mutation of the Securin gene and therefore it is believed that promoter mutation does not play any role for its enhanced expression [38, 39]. Further, no loss of heterozygosity has been reported for the region mapping the PTTG1/Securin locus [39]. In a separate study, it was demonstrated that RAN-alkaloids create more relaxed chromatin structure by altering poly-ADP-ribosylation of cellular proteins which could be considered as a causative factor for RAN-induced carcinogenesis [40]. Therefore, we have analysed the histone covalent modifications pattern both at the global level and in the promoter region of the Securin gene because such modifications do have profound effects on gene promoter activity and have been extensively linked to cancer [41]. The methylation status in a CpG island at the proximal promoter region of the PTTG1/Securin gene was not evaluated since no methylation was observed in both healthy tissues and differentiated thyroid carcinoma samples, and also in prostate cancer cell lines regardless the expression status of PTTG1/Securin gene [39].

The present results show hyperphosphorylation of Rb and upregulation of E2F1 in the RAN-treated samples and therefore such deregulation of Rb-E2F1 circuit might be involved in the upregulation of

Securin and its oncogenic role. Earlier it has been demonstrated that the expression of hPTTG1 is regulated by Rb/E2F1 pathway [22] which play an important role in carcinogenesis [42, 43]. In pituitary tumors, it has been reported that E2F1 affects PTTG1 expression [44]. Moreover, microarray analyses identified several mitotic checkpoint proteins such as Bub1, Bub3, securin, and Cdc20 are putative E2F1 targets [45].

Analysis of posttranslational histone H3 modifications both at the global level and in the promoter region of Securin gene was performed after RAN-exposure. Significant increase in tri-methylation at H3K4 residue and acetylation at H3K9 and H3K18 residues at global level in stomach tissue was noted. Trimethylation of H3 lysine4 is strongly associated with transcriptional activation and such epigenetic modification is usually observed near transcriptional start sites of highly expressed genes [46]. H3K4me3 is considered a well-established marker of active gene promoter [47] and enzyme complexes involve such modification have been implicated in oncogenesis [48,49]. In addition, changes in acetylation signalling due to mis-regulated HATs or HDACs can cause chromatin decompaction which leads to abnormal gene expression including activation of proto-oncogenes [50] as well as impair DNA damage responses [51], which together can impact genome-epigenome stability. The present ChIP-qPCR data have revealed the elevation of hypermethylation of H3K4 and hyperacetylation of H3K9 and H3K18 within promoter region of Securin gene, whereas the transcriptional repression-associated methylation like H3K9me3 [52] was reduced significantly. Increased H3K4me3 in the promoter is functionally correlated with an increase in acetylation of H3K9 and both the histone modifications are considered to be a major modification of transcribed genes [53,54]. Moreover, transcription facilitator H3K9 acetylation is mutually exclusive to the transcriptionally repressive H3K9 methylation [55]. Interestingly, the present data show reduced level of H3K9me3 correspondent to the increase in H3K9ac levels indicate that the cross-regulation between H3K9 methylation and H3K9 acetylation play in establishing a transcriptionally active state of the Securin promoter in response to RAN mediated stimulation.

These posttranslational modifications of histone are catalysed by number of enzymes and the present data clearly show higher expression of lysine-N-methyltransferase 2A (KMT2A) and lysine acetyltransferase (KAT2A) and decreased the level of HDAC3 after RAN exposure which is in support of the observed elevation in H3K4me3, H3K9ac and H3K18ac in the promoter region of Securin gene. Similar higher expression of enzymes regulating methylation and acetylation of histone in esophageal cancer of north-eastern population of India having the habit of areca-nut chewing was demonstrated [28]. In addition, present result showed an enhanced level of p300 and P300/CBP-associating factor (PCAF) after 300 days of RAN feeding. EP300, also known as KAT3B is a large, multi-domain protein, which in addition to their catalytic HAT domain, contain bromodomains that bind acetylated histones and are required for chromatin binding for transcriptional activation [56]. The PCAF is a histone acetyltransferase that primarily acetylates H3 histones and has a strong association with tumour initiation and progression [57]. It has been demonstrated that PCAF is specifically required for whereas p300 are involved in H3K18ac in cells [58].

Two sets of primers were used in the present ChIP-qPCR study, covering the upstream region of the promoter (-478 to -874) within which the core enhancer sequence critical for PTTG1 transcriptional activation is present [59]. It is worth mentioning that higher H3K9ac was observed in the upstream whereas elevation of H3K4me3 and H3K18ac was observed in the downstream region of the selected portion of the promoter. It seems that KAT2A complex recruited to an upstream element and catalyzed local H3K9 acetylation which disrupts the interaction between the tail and the negatively charged nucleosomal DNA to facilitate opening of chromatin and attract bromodomain-containing transcription factors like EP300 that would acetylate histones in neighbouring nucleosomes, which would stimulate HAT binding to the newly acetylated site and promote active transcription [60,61].

It is interesting to note that RAN-exposure hyperphosphorylates Rb and thus allowing E2F1 to drive transcription of its target genes including PTTG1/Securin. Although, pRB/E2F pathway have been extensively studied for decades but its involvement in other non-canonical functions is emerging through its interaction with chromatin modifier proteins [62]. Therefore, it might be possible that the present histone3 acetylation at K9 and K18 could be facilitated by inactivated pRb as it was demonstrated in DNA break repair [63]. In addition, pRb also regulates H3K4methylation by inhibiting demethylase action of KDM5A through its interaction [64]. Thus, in a normal cell pRb, a putative tumor suppressor protein, is not only repressing transcription by inhibiting E2F family of transcriptional factors but also playing a pivotal role in the maintenance of the chromosome structure and stability via physical interactions with chromatin-related proteins to silence transcription [65]. It seems that RAN-exposure leads to pRb hyperphosphorylation which subsequently develop chromosomal instability (CIN) and aneuploidy as it was reported earlier [66]. It has been demonstrated that induction of CIN and overexpression of Securin are significantly associated with the gastric cancer in mice [6] and both oral and esophageal cancers in human with RAN consumption habit [7)]. Therefore, the results of the present study have led to the hypothesis that Securin overexpression and subsequent elevation of CIN and aneuploidy are probably a by-product of the inactivation of the pRB pathway. Support for this idea comes from several earlier studies that had shown the expression signature of more than 10 genes, including PTTG1/Securin, that show strong correlation with CIN are putative E2F targets, and are surrogate markers of pRb inactivation [67,68]

Conclusions

This study shows that overexpression of Securin after RAN-exposure could be due to deregulation of Rb-E2F1 circuit and subsequent elevation of H3K4me3, H3K9ac and H3K18ac in the promoter region of the Securin gene. However, RAN-mediated deregulation of E2F cell cycle targets including Securin concurrent with loss of genome stability hindered the ability to assess whether pRB could maintain genome integrity independent of E2F transcriptional control. Further in-depth research is required to understand how many cellular processes are altered when RB1 is mutated. Expanding our understanding on how epigenetic modifications contribute to oral, esophageal and gastric cancers in RAN-users may not only improve the knowledge of pathogenesis but also provide new novel biomarkers for diagnosis or disease outcome prediction and/or response to therapy.

Abbreviations

RAN: Raw areca-nut; PTTG1: pituitary tumor transforming gene 1; CIN: Chromosomal instability; pRb-protein retinoblastoma; H3K4me3**:** Histone H3 Lysine 4 trimethylation; H3K9ac: Histone H3 Lysine 9 acetylation; ChIP: chromatin immunoprecipitation; KMT2A: lysine methyltransferase2A; KAT2A: lysine acetyltransferase2A; KDM4C: histone demethylase; HDAC3: histone deacetylase3; PCAF: P300/CBP-associating factor

Declarations

Ethics approval

The present experiments were conducted in compliance with institutional guidelines and approved by our “Institutional Ethical Committee for Animal Care and Use” Board, in the School of Life Sciences, North-Eastern Hill University, Shillong, India (IECAC/2014/07).

Consent for publication

All authors agree to the publication of the article.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

All the authors declare that they have no competing interests.

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Author Contributions

AC and NB conceived and designed the study. NB, CSS and PS performed the experiment and all together analysed the data. HD and HBN analysed the histopathological data. AC wrote the manuscript and other co-authors contributing to editing the manuscript

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Figures

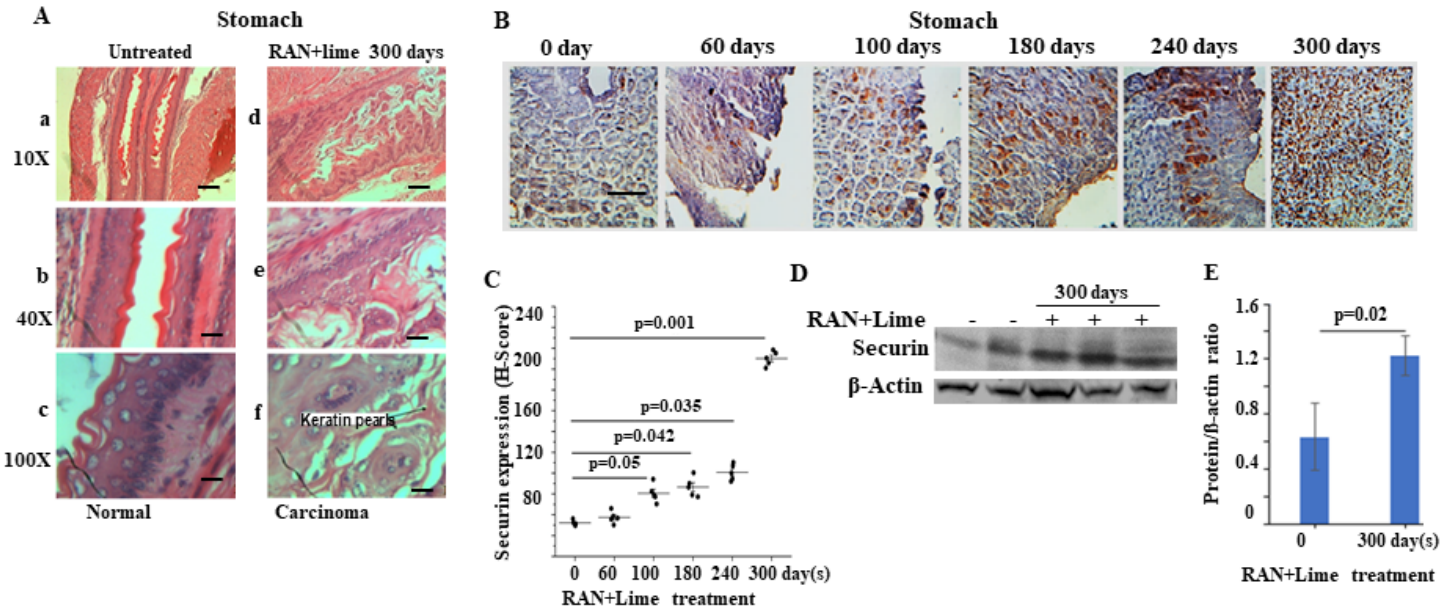


Figure 1

Expression analysis of mouse PTTG1/Securin gene in both normal and tumor tissues following treatment with RAN+lime. A. Histopathology of untreated normal and tumorous stomach of mice following RAN+lime treatment for 300 days (scale bars: 200 µm). Carcinoma shows squamous keratin pearls. The magnification is indicated as 10X, 40X and 100X. B. Immunohistochemical images of mouse stomach treated with RAN+lime for various duration. The normal expression of Securin gene in untreated control and gradual upregulation of Securin expression in treated samples for different time periods are shown. The magnification of all these images is x40 and the scale bar: 200 µm. The expression level of Securin in untreated and treated mice was analysed by H-score and are shown the mean H-score ± SEM in C. D. Representative western blotting detection of Securin and ß-actin in mouse stomach cells after exposure with RAN+lime for 0 and 300 days. E. The quantitative densitometric analysis of the level of proteins of the genes mentioned in D. The values are the mean±SEM of the number of individuals used in

this experiment. The values are normalized to respective ß-actin values. In both C and E, the P values are shown compared with untreated control (as determined by paired t-test). P values less than 0.05 are considered significant.

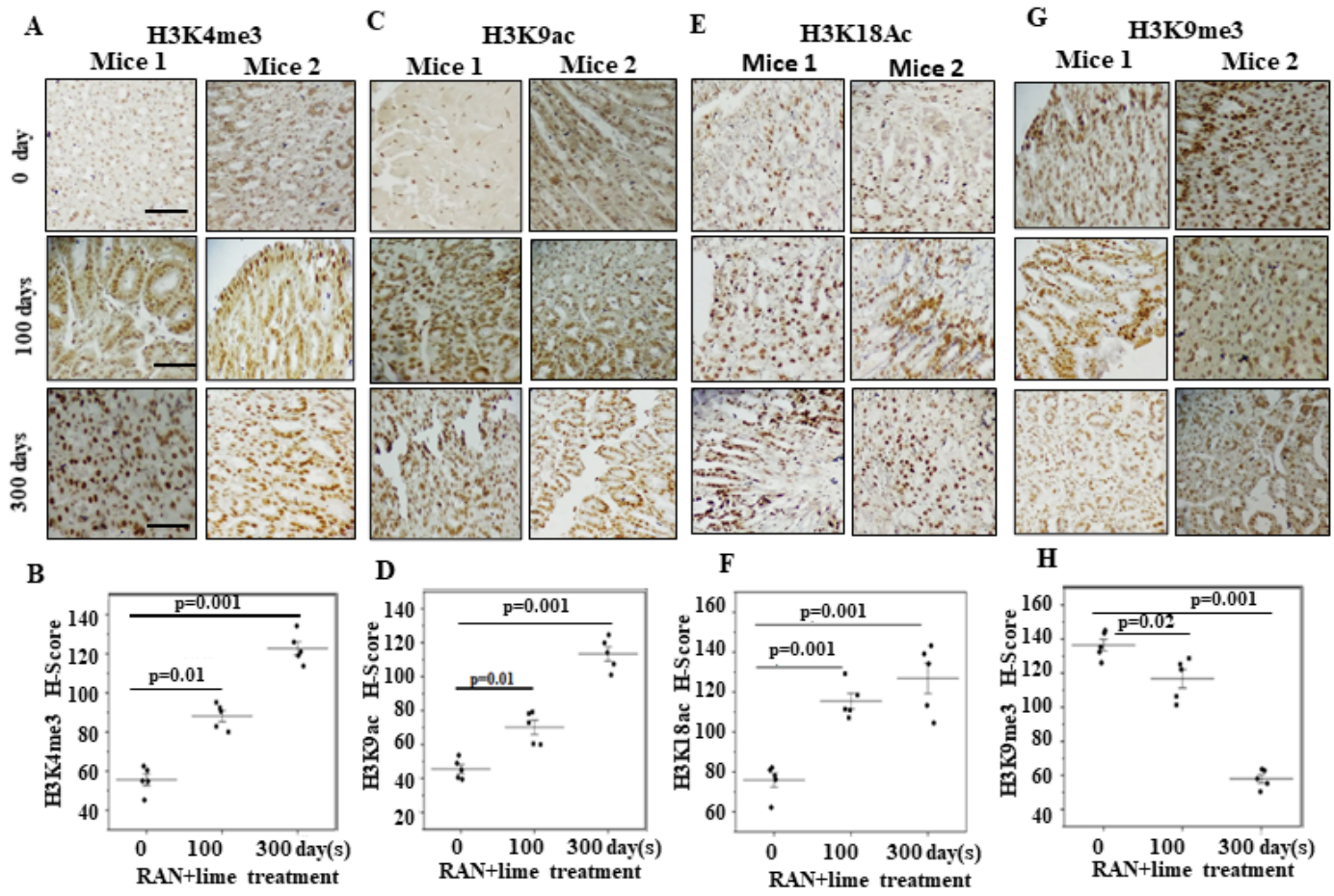


Figure 2

Immunohistochemical images for Histone H3 methylation and acetylation in mice stomach cells. A. Representative images show H3K4me3 positive cells increase with the treatment duration. B. H-scores based on IHC for H3K4me3 positive cells. C. Representative images show H3K9ac positive cells increase with the treatment duration. D. H-scores based on IHC for H3K9ac positive cells. E. Representative images show H3K18ac positive cells increase with the treatment duration. F. H-scores based on IHC for H3K18ac positive cells. G. Representative images show H3K9me3 positive cells decrease with the treatment duration. H. H-scores based on IHC for H3K9me3 positive cells. The magnification of all these images is 40x. Data were analysed using one way ANOVA with Turkey's multiple comparison post-tests. In each case images of two mice were shown. The scale bar: 200 µm.

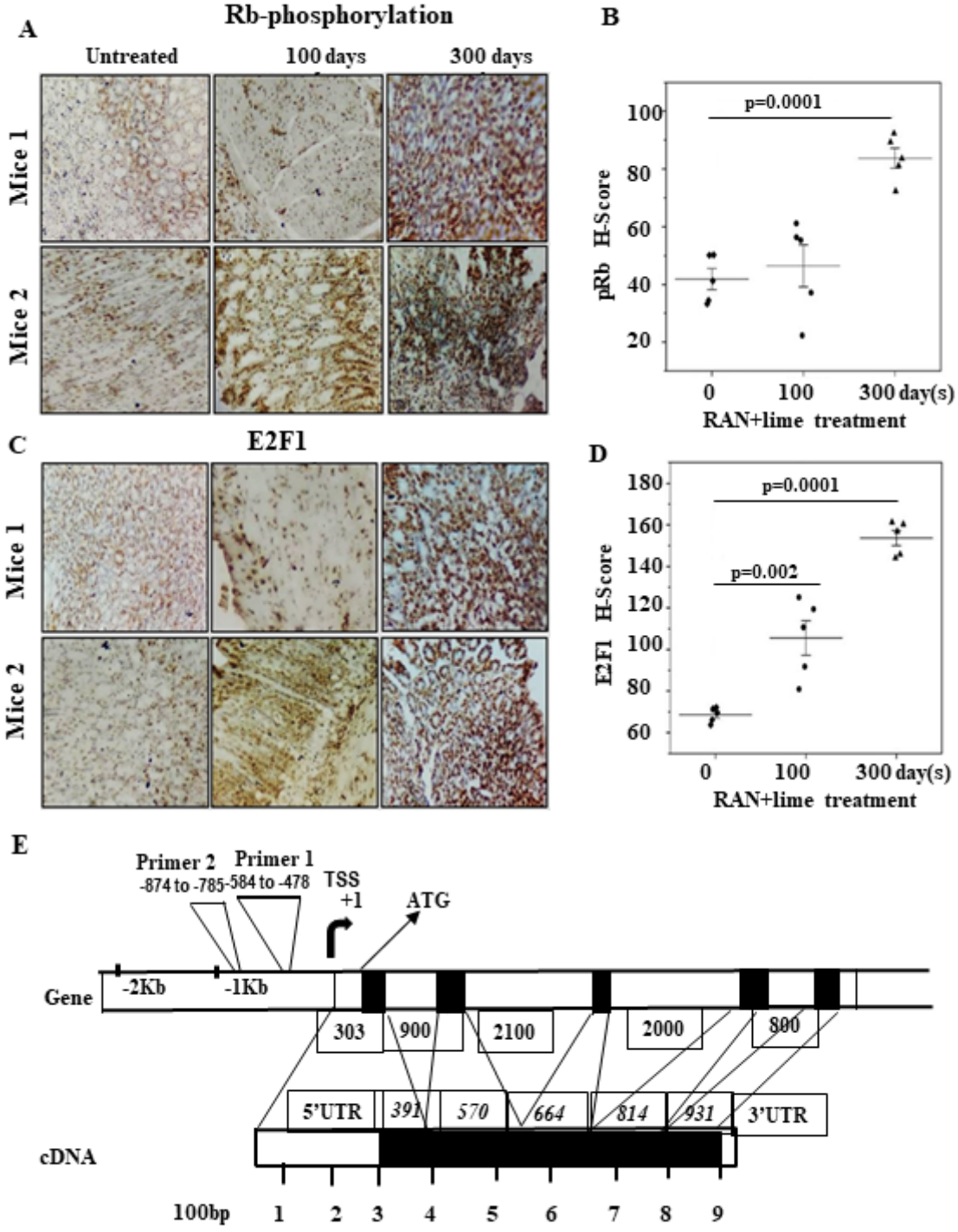


Figure 3

Immunohistochemical images for Rb-phosphorylation and E2F1 expression and Securin gene structure. Immunohistochemical assays of A. phosphor-retinoblastoma (pRb) and C. E2F1 expression in mice stomach cells with and without RAN+lime treatment. Quantitative values are represented in B and D as mean ± SEM; P-values were calculated with untreated control using one-way ANOVA with Turkey's multiple comparison post-tests. In each case images of two mice were shown. The scale bar: 200 μm. E. Schematic illustration of mouse PTTG1/Securin gene structure. Exons are indicated as boxes (translated

sequences in black and untranslated regions in white). Exon and intron sizes (bp) are indicated. The transcription initiation site (TSS) is shown as +1 and the translation start site ATG is indicated with an arrow. The promoter region shows in Kb and the region is highlighted from where the primer pair was designed. The lower panel represents cDNA with the scale multiple of 100bp. Both 5- and 3-flanking untranslated regions are shown.

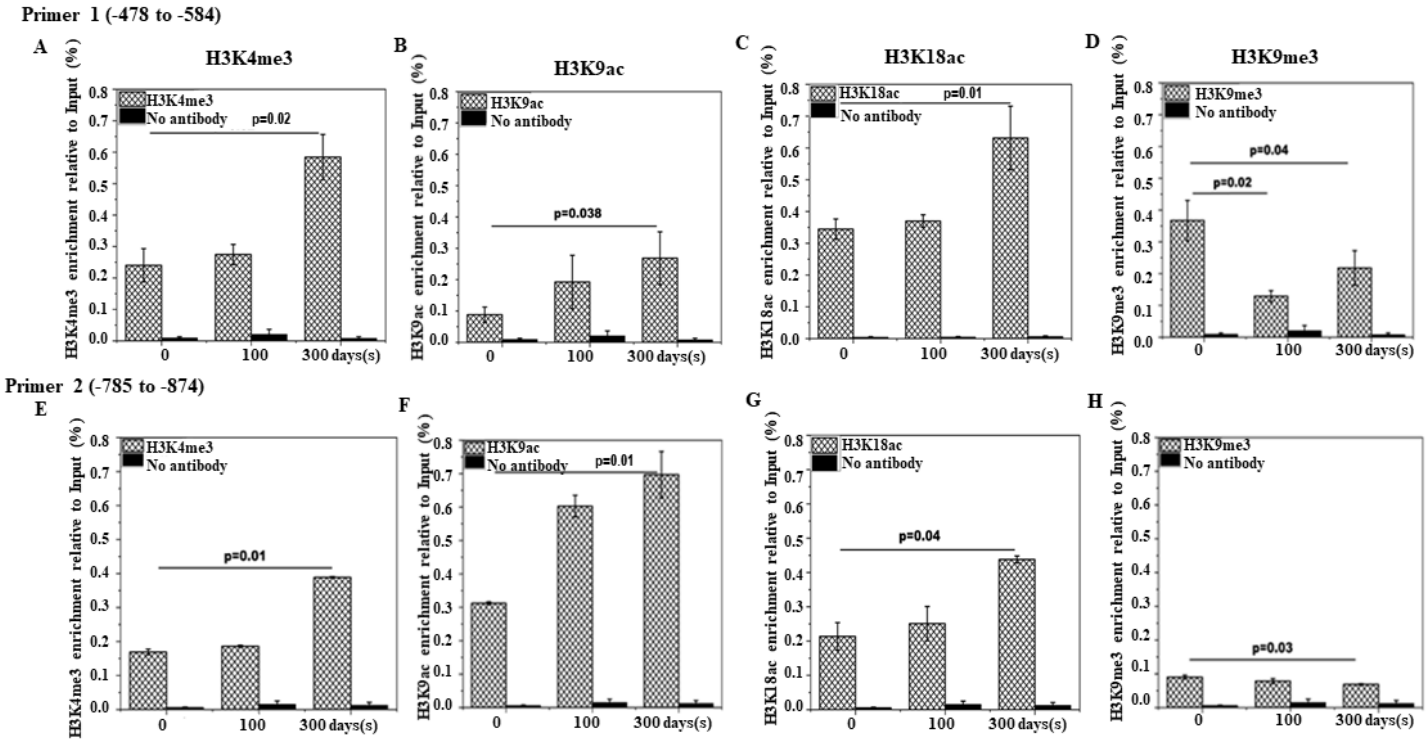
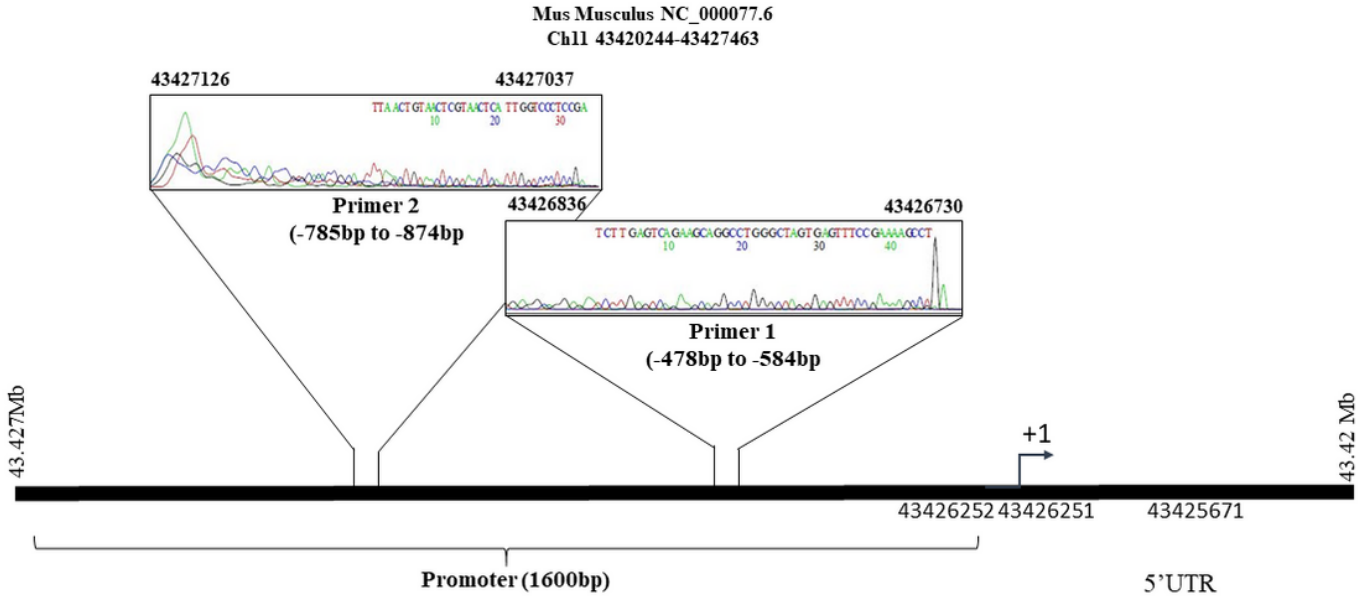


Figure 4

ChIP analysis of Histone H3 methylation and acetylation at the promoter of Securin gene. ChIP-qRT-PCR assays for H3K4me3, H3K9ac, H3K18ac and H3K9me3 recruitment to the two regions of Securin promoter -478 to -584 (upper panel A-D) and -785 to -874 (lower panel E-H) in mice stomach cells treated with RAN+lime for 0, 100 and 300 days. Chromatin was cross-linked, fragmented and immunoprecipitated with no antibody (as negative control) or anti-H3K4me3, H3K9ac, H318ac and H3K9me3 ChIP-grade antibody and the purified DNA was used to amplify with two sets of primer pairs of two regions (-478 to -584 with Primer 1; -785 to -874 with Primer 2) of Securin promoter in qPCR. As Input, 10% diluted chromatin fragments were kept and used in qPCR for the enrichment analysis. Percent of input values represent the mean of four different animals ± SEM. Data were analysed using one way ANOVA with Turkey's multiple comparison post-tests. P values less than 0.05 are considered significant.



Mouse PTTG1 Primer 2 location :chr11.43427037-43427126
(CAGGCCATCCTGGTCTACATAGTGATTCCCAGGACAGCCAGGGT
CTTCTTCAAGATCAGTAAGTGCTTGTAACTGCCCAGGAATCTCTC
C) **Product size**: 90bp

Mouse PTTG1 Primer 1 location : chr11:43426730-43426836
(GGCCTGTTCCCCTAGAGATTACACTCTGGCTACTCTGATCTGAGCTC
TGAGGCAGGAGAATCTTGAGTCAGAAGCAGGCCTGGGCTAGTGAG
TTTCCGAAAAGCCTG) **Product size**: 107bp

Figure 5

Sequencing of qRT-PCR products: Schematic diagram depicting the position of qRT-PCR product which was sequenced and matched in the promoter region (-478 to -584 amplified by Primer 1; and -785 to -874 amplified by Primer 2) of Securin gene in mouse. The transcription initiation site (+1) is indicated with an arrow. The sequence details of both the amplified products are shown below.

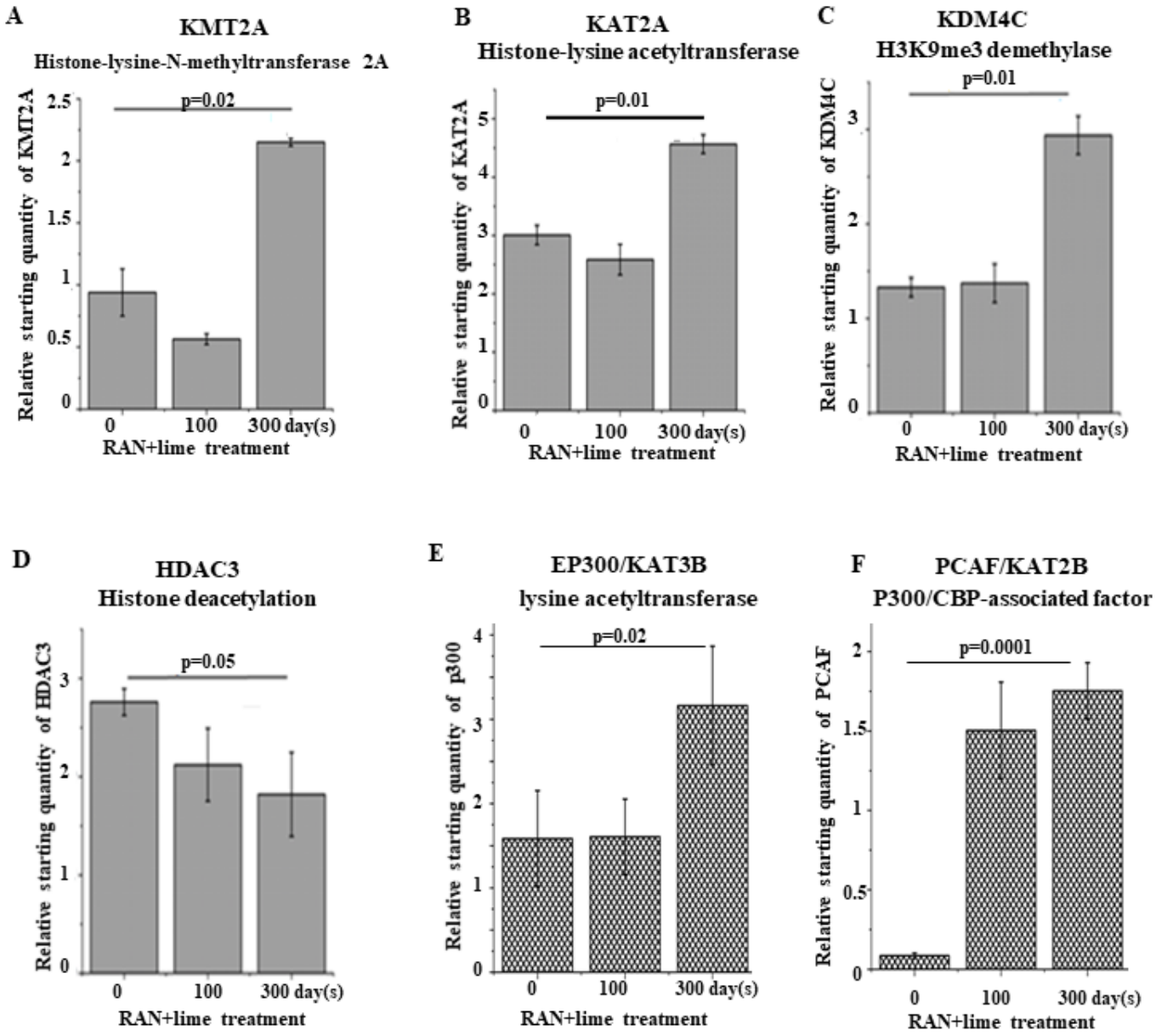


Figure 6

Expression of epigenetic chromatin modification enzymes after RAN treatment. Stomach cells of mice treated with RAN+lime for 0, 100 and 300 days and analysed by qRT-PCR for A. Histone-lysine-N-methyltransferase2A (KMT2A), B. Histone acetyltransferase (KAT2A), C. demethylase (KDM4C), D. Histone deacetylation (HDAC3), E. EP-300/KAT3B and F. PCAF/KAT2B genes expression. Data are the mean±SEM of a representative experiment performed in untreated control (N=4) and treated (N=4 in each category). The values are normalized to respective GAPDH values. Data were analysed using one way ANOVA with Turkey's multiple comparison post-tests. P values are shown in all and considered significant when the values are less than 0.05.

Supplementary Files

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