DOI: https://doi.org/10.53350/pjmhs22162512

ORIGINAL ARTICLE

Contribution of Long Non-Coding Rnas (Ccat1, Malat1, and Pandar) in the Pathogenesis of Colorectal Cancer

AYAT B. AL-GHAFARI[1,2], HALIMA A. SIDDIQUI[1], HANI M. CHOUDHRY[1,3], HUDA A. AL DOGHAITHER[1], HUDA F. ALSHAIBI[1], HADEIL M. ALSUfiANI[1], SAWSAN A. RAHIMULDDIN[1]

[1]Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

[2]Cancer and Mutagenesis Unit, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

[3]Center for Artificial Intelligence in Precision Medicines, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

Corresponding author: Ayat B. Al-Ghafari, Email: abalghafari@kau.edu.sa, Cell: +966504686549, ORCID: 0000-0001-9156-4263

ABSTRACT

Objectives: To assess the relationship between the expression of long non-coding RNAs (lncRNAs), CCAT1, MALAT1, and PANDAR, in CRC patients with varying physical, environmental, and pathophysiological factors.

Material and Methods: A case-control study was conducted at King Abdulaziz University (Jeddah, Saudi Arabia). Whole blood was collected from 63 CRC patients and 40 healthy subjects in the period from January to August 2016. Anthropometric measurements were taken and the total RNA was isolated and the expression of lncRNA was determined by real-time polymerase chain reaction. Various software programs, including REST2009 and GraphPad Prism version 7, were used to analyze the different statistical correlations.

Results: MALAT1 was significantly highly expressed in CRC female patients, whereas CCAT1 and PANDAR were significantly up-regulated in male CRC patients, as compared with controls. Moreover, in obese CRC patients, CCAT1, MALAT1, and PANDAR expressions were significantly up-regulated, while in overweight CRC patients, only MALAT1 was significantly more highly expressed, compared to controls. There was a slight increase in PANDAR expression in high-grade CRC patients, compared to low-grade patients. Finally, CCAT1 was significantly up-regulated in high fish intake CRC patients and was significantly correlated with smoking status.

Conclusion: LncRNAs may contribute to CRC pathogenesis by affecting several physical and environmental factors.

Keywords: Colorectal cancer, LncRNA CCAT1, LncRNA MALAT1, LncRNA PANDAR

INTRODUCTION

Worldwide, cancer is one of the major leading causes of death. Among human cancers, colorectal cancer (CRC) is considered the third cancer with high incidence rate after lung and breast and the second most common cancer with high mortality rate after lung cancer.[1] In Saudi Arabia, according to the Saudi national cancer registry (NCR), CRC ranks the first and the third most common cancer among males and females, respectively, with 1659 cases diagnosed in 2016.[2] It is important to notice that it is difficult to diagnose CRC in Saudi population for many reasons. First, most of the Saudis are classified in the younger age class (35% in the age group 20–39 years and 86% ≤ 50 years old). Secondly, obesity is common among adult Saudis with a trend of (35%).[3] As a consequence, many CRC patients die at the advanced stage of the disease.[4] Therefore, an urgent need for more research is required to identify new biomarkers for tumor prognosis and diagnosis.

Tumor biomarkers show great promise in the diagnosis of cancer, but only a few biomarkers have been discovered for use in clinical practice.[5] Non-coding RNAs (ncRNAs) are RNA transcripts that have RNA and protein – like functions. Long non-coding RNA (lncRNA) is a subclass of ncRNAs with more than 200 nucleotides and have no capability for coding proteins.[6] They are majorly expressed in nucleus but some of them can be found in cytoplasm.[7] So far, more than 60,000 lncRNAs have been discovered in human.[8] Since their discovery, they were found to play a role in many normal and abnormal physiological processes such as genes transcription regulation,[9] chromatin alteration,[10] DNA epigenetic regulation, and histone methyltransferases regulation, thereby possibly contributing to the maintenance and the development of many diseases.[11]

Abnormal expression of lncRNAs was involved in the pathogenesis of many human cancers due to their interactions with DNA, cellular proteins, epigenetic genes, as well as in other cellular mechanisms such as cells differentiation, proliferation, invasiveness, and apoptosis.[12-14] Therefore, they may be considered as a promising cancer biomarkers, drug targets, and prognostic factors.[15]

LncRNA colon cancer-associated transcript-1 (CCAT1) was shown to be highly expressed in cancers such as gallbladder, hepatocellular, and all stages of CRC adenomas and adenocarcinomas, therefore, it serves as a key target for real-time in vivo imaging.[9,16] In contrast, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is essential in (G1/S) phases mitotic regulation and in some cancers, it was found to be over-expressed.[17] In addition, the lncRNA known as the promoter of CDKNIA antisense DNA damage-activated RNA (PANDAR) can decrease pro-apoptotic genes expression via its interaction with nuclear transcription factor Y subunit alpha (NF-YA). Its production was found to be induced by p53 as a result of cellular DNA damage, and its knockdown increases the DNA damage-induced apoptosis.[15,18]

In this study, the relationship between the expressions of three selected lncRNAs—CCAT1, MALAT1, and PANDAR—in the blood samples of healthy individuals and CRC patients, with different physical characteristics, such as age, gender, body mass index (BMI), and other pathophysiological features of CRC, such as drug

response, clinical grades and stages, were assessed to evaluate the possible effects of those three lncRNAs on the pathogenesis of CRC.

MATERIALS AND METHODS

Materials: Total RNA was extracted from whole blood samples using QIAGEN-QIAamp RNA blood mini kit (52304: Qiagen, Germany). The cDNA samples were prepared from total RNA samples using High-Capacity cDNA Reverse Transcription Kit (4368814: Thermo Fisher Scientific, Lithuania) and RNase Inhibitor reagent (N8080119: Thermo Fisher Scientific, USA). Quantitative real time polymerase chain reaction (qRT-PCR) was performed using Universal 2x PowerUP™ SYBR® Green Master Mix Kit (A25741: Thermo Fisher Scientific, USA).

Study design: The study protocol was approved by the local Institutional Review Board (King Abdullah Medical City in Holy Capital, Saudi Arabia) under reference no. (IRB-002-FR1). All participants signed an informed consent form that follows the declaration of Helsinki and filling out a questionnaire containing information about their anthropometric measurements as well as smoking and nutritional status. From each participant, an EDTA blood sample was collected for RNA gene expression analysis. The study was conducted on (n = 63) CRC patients from Oncology Centers at King Abdullah Medical City (KAMC) in Holy Capital (Makkah), Saudi Arabia. The patients were selected based on their confirmed biopsy- or colonoscopy neoplasm report and a documented clinical history. On the other hand, control blood samples were collected from (n = 40) healthy blood donors from blood bank at King Fahad General Hospital, Jeddah, Saudi Arabia. The healthy control subjects were selected based on their clinical examination and laboratory investigations (free of any signs of inflammation or malignancy).

RNA extraction and quality control: Before performing real time PCR, the erythrocytes in the collected whole blood samples were lysed and centrifuged to recover leukocytes. Then, using highly denaturing conditions, total RNA was isolated by lysing leukocytes and inactivating RNases. Several homogenization steps were performed using ethanol and spinning columns. Finally, RNA (50 µl) was extracted using washing buffers and was eluted with RNase free water. The concentration and quality of each extracted RNA sample was determined using Nanodrop spectrophotometer ($\lambda_{260/280}$). The extracted and purified RNA samples were kept immediately in -80°C to prevent degradation.

Quantitative real-time polymerase chain reaction (qRT-PCR): To reveal the expression level of lncRNAs, the qRT-PCR was performed. First, the extracted mRNA (300 ng) was reversed transcribed into cDNA (20 µl) following manufacturer's protocol without any modification. All purified cDNAs samples were kept in -20°C for future experiments. The expression level of CCAT1, MALAT1, and PANDAR was measured by qRT-PCR using the SYBR Green protocol. RPL11 expression level was used as a housekeeping gene control. The primers and qRT-PCR conditions used for the quantification of the three lncRNAs were as previously described in[19].

Statistical analysis: The statistical analyses were made by GraphPad Prism version 7.00 (San Diego California, USA) and REST 2009 software. Unpaired t test was used to compare the differences between two independent groups. One way analysis of variance (1-way ANOVA) was applied to compare between three or more independent groups and P values were statistically corrected using Bonferroni's post-hoc test. Data were expressed as the mean ± standard error of the mean (SEM). For gene expression analysis, the relative expression levels (fold change) as well as P values were calculated using REST 2009 software. Then, the expression levels of CCAT1, MALAT1, and PANDAR were normalized to the expression of RPL11. P value ≤ 0.05 was considered statistically significant.

RESULTS

According to their gender, patients and controls physical characteristics were divided into two groups (male and female). For CRC patients (n = 63), 48 (76.2%) were males and 15 (23.8%) were females. In CRC patients, unpaired t test results showed a highly significant difference in height between males and females (Table 1). The control group (n = 40) was likewise divided into two groups: 29 males (72.5%) and 11 females (27.5%). Unlike patients, as shown in Table 1, significant differences were observed in many physical characteristics such as height, BMI, and waist to hip ratio (WHR) between males and females in the control group.

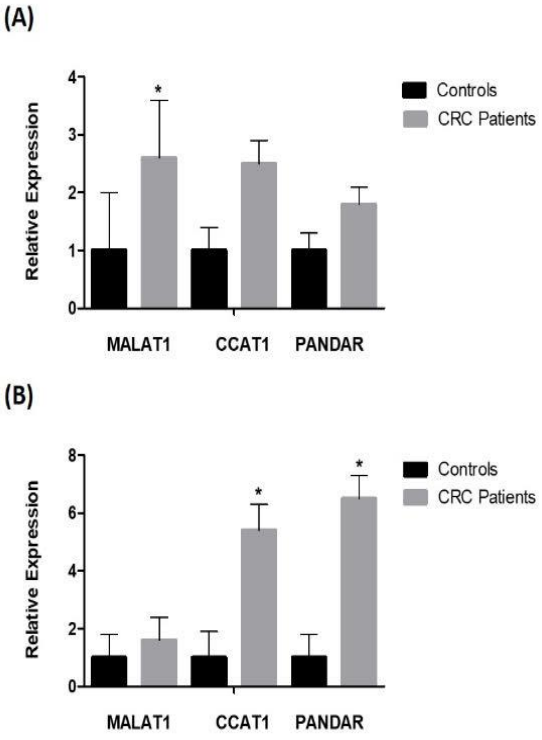

Figure 1: The association of gender with relative expression of lncRNAs (MALAT1, CCAT1, and PANDAR). As shown by the figure, MALAT1 was significantly expressed in female patients compared to female controls (A). However, CCAT1 and PANDAR showed a higher significant expression in male CRC patients compared to male controls (B) (*P < 0.05).

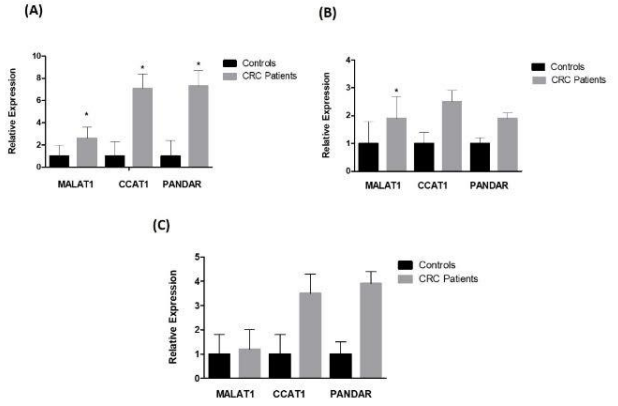

Figure 2: The association of BMI as obesity index with relative expression of lncRNAs (MALAT1, CCAT1, and PANDAR). The patients and controls were divide into four groups according to their calculated BMI. None of the controls were categorized as lean. The comparison of relative expression of lncRNAs (MALAT1, CCAT1, and PANDAR) showed different correlations with different BMI scores. The three lncRNAs expressions were significantly higher in patients (*P < 0.05) than in controls in the obese category (A). Whereas, in overweight category, only MALAT1 had a significant difference in the expression (*P < 0.05) (B). Finally, none of the three lncRNAs showed a significant difference (P > 0.05) in the normal body weight category (C).

The mean difference analysis using unpaired t test showed that CCAT1 and MALAT1 had a significant difference in their mRNA expression levels between patients and controls (CCAT1 mRNA level in CRC versus controls was 26.83 ± 0.136 vs. 27.30 ± 0.168 and P value = 0.04; whereas, MALAT1 mRNA level in CRC versus controls was 19.41 ± 0.197 vs. 18.59 ± 0.142 and P value = 0.0029). However, the qRT-PCR raw data that were previously analyzed on REST 2009 software for the three lncRNAs revealed that the relative expressions (fold-change) of CCAT1, MALAT1, and PANDAR were significantly over-expressed in CRC patients compared to healthy control subjects (P ≤ 0.05) [the fold-change for CCAT1 = 4.54, MALAT1 = 1.86, and PANDAR = 4.68].[19] Therefore, these three lncRNAs were selected for further analyses and correlations with other important anthropometric and clinicopathological features of CRC.

Regarding age, CRC patients were divided according to the mean age (56 years) into (n = 38, 60.32%) under age 56 and (n = 25, 39.68%) above the mean. However, the comparison by unpaired t test did not show any significant difference in the mean of the relative expression level (fold-change) between the patients' age and the three lncRNAs (P > 0.05). Moreover, according to their gender, patients were divided into (n = 15, 23.8%) CRC females and (n = 48, 76.2%) CRC males. Although the statistical comparison showed that there was no significant difference between the mean of each lncRNAs between male and female CRC patients, in female patients, the relative expression of CCAT1 (2.5, P = 0.07), MALAT1 (2.6, P = 0.004), and PANDAR (1.8, P = 0.3) were up-regulated. However, only MALAT1 relative expression showed significant P value. In contrast, in CRC male patients, the correlation comparisons of relative expression levels, revealed that CCAT1 (5.4, P = 0.0001) and PANDAR (6.5, P = 0.0001) were expressed higher than in female CRC patients, whereas, MALAT1 (1.6, P = 0.05) was significantly expressed at lower level in CRC males compared to CRC females [Figure 1].

Table 1: Anthropometric characteristics of the study participants

Anthropometric characteristic	Patients (n = 63) Mean ± SEM			Controls (n = 40) Mean ± SEM		
	Males (n=48)	Females (n=15)	P value	Males (n=29)	Females (n=11)	P value
Age (years)	56.25 ± 1.69	56.93 ± 3.68	0.75	51.86 ± 1.69	50.45 ± 2.85	0.76
Height (cm)	167.90 ± 1.31	157.30 ± 1.37	≤ 0.0001***	168.8 ± 1.39	158.2 ± 1.76	≤ 0.0001***
Weight (kg)	76.21 ± 2.22	66.34 ± 3.77	0.05	86.03 ± 3.12	88.73 ± 4.81	0.72
Waist (cm)	101.0 ± 2.99	100.0 ± 4.68	0.88	106.2 ± 3.86	98.00 ± 4.87	0.28
Hip (cm)	110.90 ± 2.75	110.3 ± 4.81	0.94	103.7 ± 3.51	110.3 ± 4.34	0.22
WHR	0.9165 ± 0.02	0.9153 ± 0.04	0.42	1.035 ± 0.03	0.887 ± 0.02	≤ 0.0001***
BMI (kg/m^2)	27.12 ± 0.83	26.79 ± 1.47	0.94	30.29 ± 1.15	35.54 ± 1.96	0.03*

*P < 0.05; ***P < 0.001; WHR: waist to hip ratio; BMI: body mass index

According to the WHO classification of BMI, CRC patients (n = 63) were divided into four main groups as follows: lean (n = 5, 8%), normal weight (n = 21, 33.3%), overweight (n = 15, 23.8%), and obese (n = 22, 34.9%). On the other hand, the controls (n = 40) were classified into three groups: normal weight (n = 6, 15%), overweight (n = 13, 32.5%), and obese (n = 21, 52.5%); no control participant fell in the lean category. Results in [Figure 2] showed that obese CRC patients had significantly higher expression levels (P ≤ 0.0001) of the three lncRNAs compared to obese controls [these levels were as follows: CCAT1 (7 fold change), MALAT1 (2.5 fold change), and PANDAR (7.3 fold change)]. However, in overweight CRC patients, results showed that only MALAT1 was significantly up-regulated (P ≤ 0.05). Interestingly, the significant expression of the three lncRNAs was not observed in the lean and normal BMI CRC patients (P > 0.05).

Patients were categorized into four clinicopathological stages: stage I (n = 7, 11.11%), stage II (n = 9, 14.28%), stage III (n = 16, 25.40%), and stage IV (n = 31, 49.21%)] following the tumor-node-metastases (TNM's) classification system. Expression levels of three lncRNAs were compared in low-grade CRC stages (stage I versus stage II). The results showed non-significant fold change difference between relative expression levels and

progression for the three lncRNAs [CCAT1 (0.9, P = 0.9), MALAT1 (1.2, P = 0.5), and PANDAR (1.6, P = 0.7)]. Furthermore, the relative expression of the three lncRNAs was also compared in high-grade CRC stages (stage III versus stage IV). The results also showed a non-significant difference [CCAT1 (0.9, P = 0.9), MALAT1 (1.3, P = 0.5) and PANDAR (0.8, P = 0.7)]. CRC patients were grouped into grade I (low-grade), grade II (moderate-grade), and grade III and IV (high-grades) to elucidate the effect of lncRNA relative expressions on cancer grading. CRC patients with grade A of stages II, III, and IV were compared with grade B of stage III and IV. The expression level for CCAT1 and MALAT1 was not significantly difference [1.7 (P = 0.3) for CCAT1 and 1.4 (P = 0.5) for MALAT1]. However, a slight increase for PANDAR in high-grade CRC patients (i.e., grade B of stage III and IV) was observed (fold change was 2.1, P = 0.2).

The expression of the three lncRNAs in CRC patients was compared to different environmental factors. The first examined environmental factor was food consumption particularly vegetables and fish intake. The CRC patients were categorized into low and high vegetables intake. The expressions of those three lncRNAs did not show any significant changes with vegetables intake. The relative expression was 1.2 (P = 0.6), 0.9 (P = 0.8), and 2.1 (P = 0.2) for CCAT1, MALAT1, and PANDAR, respectively. Furthermore, following the same manner as above, CRC patients were grouped into low and high consumers according to their fish consumption. The expressions for the three lncRNAs in the low fish consumption group in comparison to high fish consumption group were 2 (P = 0.02), 1.2 (P = 0.5), and 1.2 (P = 0.7) for CCAT1, MALAT1, and PANDAR, respectively. Interestingly, results showed that only CCAT1 was significantly up-regulated in high fish intake CRC patients.

The other environmental factor was smoking. CRC patients were categorized into nonsmokers (n = 39, 61.9%) and smokers (n = 24, 38.1%). Results showed that only CCAT1 had a significant change in relative expression level between the two groups (fold change 2.1, P = 0.03). The other two lncRNAs MALAT1 and PANDAR had no significant relation with smoking (fold change for both lncRNAs was 1.2, P = 0.6).

DISCUSSION

CRC has a higher incidence rate worldwide being higher in males compared to females.[1] There are many reasons for poor survival rate for CRC patients, most commonly the late diagnosis for many CRC cases. Therefore, there is a rationale to develop new diagnostic methods that could be more effective, cheaper, and easy to perform.[15,19] LncRNAs were found to be released in the plasma of cancer patients. Hence, they could be promising monitoring biomarkers for cancers diagnosis and treatment.[5] According to these point of views, this study was designed to seek the relationship between expression levels of three lncRNAs namely CCAT1, MALAT1, and PANDAR in CRC patients' blood samples with different physical, environmental and pathological features. Those relations, if any present, could act as biomarkers for CRC diagnosis or prognosis. The expressions of lncRNAs were widely studied and they showed a variable expression levels between normal and cancerous tissues. Interestingly, they also showed remarkable expression fold changes among different cancer types including CRC. In case of CRC, dysregulated lncRNAs showed a promising correlation with CRC treatment.[20] Moreover, these oncogenic lncRNAs could be easily detected in blood samples of cancer patients, and therefore, can be used to distinguish patients at early stages very specifically and with higher sensitivity and accuracy rate from healthy subjects.[21]

Interestingly, one of our published reports presented that CCAT1, MALAT1, and PANDAR were significantly up-regulated in the blood of CRC patients, with 4.54, 1.86, and 4.68-fold increases, respectively, in CRC samples when compared with healthy subjects.[19] For this study, further analyses were performed on REST 2009 software using volunteers' information to reveal possible correlations between the significant expression of CCAT1, MALAT1, and PANDAR on different anthropometric (age, gender, and BMI) and clinical (grades and stages) features of CRC. To the best of our knowledge, this is the first study that correlates the expression of those three lncRNAs with different anthropometric measurements as well as clinicopathological features in CRC patients. Therefore, it was not possible to compare or contrast our results against other conducted studies. Current results showed that none of the three lncRNAs had a significant correlation with age. However, regarding gender, results showed that lncRNA (MALAT1) was highly expressed in CRC female patients compared with female controls. On the other hand, the other two lncRNAs (CCAT1 and PANDAR) showed significant higher expression in male CRC patients compared with male controls. A meta-analysis study on 7 different cancers found a high expression of a newly-discovered lncRNA called a bladder cancer-associated transcript 1 (BLACAT1). This lncRNA showed a correlation between poor prognosis and overall survival rate, tumor grade, and metastasis. However, it was not significantly correlated with other anthropometric measurements such as age and gender or environmental factors such as smoking status.[22]

Moreover, as physical inactivity and poor life style that lead to obesity are considered a risk factor for CRC, the effect of the three lncRNAs expressions was compared with BMI in our study population. Results showed that only obese CRC patients had higher expression levels of the three lncRNAs when compared to the controls. Whereas, overweight CRC patients, only MALAT1 had up-regulated expression level. In fact, less is known about the correlation of lncRNAs with lipid metabolism and adipogenesis, although many studies showed a significant effect of some lncRNAs in the metabolic homeostasis in different metabolic conditions. Sun et al.[23] found that obese patients had decreased expression levels of some blood lncRNAs (p5549, p21015, and p19461) compared to non-obese patients. Furthermore, these lncRNAs had a negative correlation with important parameters for insulin sensitivity such as BMI, waist circumference, WHR, and fasting insulin. A number of obesity-associated lncRNAs such as non-coding RNA in the INK4 locus (ANRIL), H19, and HOX transcript antisense RNA (HOTAIR) were found to be dysregulated in obese subjects, suggesting that, these lncRNAs may promote different cancers through different

mechanisms.[24] Interestingly, this is the first study that correlates lncRNAs particularly (CCAT1, MALAT1, and PANDAR) expressions with BMI in CRC patients. Therefore, it was not possible to correlate and explain this correlation. In addition to the previous comparisons, CCAT1, MALAT1, and PANDAR expressions were correlated with environmental conditions such as food intake (vegetables and fish) and smoking. Only CCAT1 was significantly up-regulated in high fish intake patients as well as in smoker CRC patients. However, in other studies on lncRNAs expressions and food intake, most articles dealt with the role of antioxidants and phytochemicals with lncRNAs expression in many diseases including cancers, by studying their involvement in carbohydrate and lipid metabolism.[25] Regarding the correlation of CCAT1, MALAT1, and PANDAR with clinicopathological grading and staging of CRC, the comparison made in this study showed that high-grade CRC patients had a slight increase in PANDAR expression. In agreement with our results, Lu et al.[26] found that CRC patients with higher expression level of PANDAR characterized with poorer overall survival than patients with lower PANDAR expression.

CONCLUSION

To the best of our knowledge, this is the first report to study the effect of significant expression of the lncRNAs CCAT1, MALAT1, and PANDAR on other features of CRC patients, including physical, environmental, and clinical factors. Interestingly, the current study found significant relationships between the expression of CCAT1, MALAT1, and PANDAR with BMI, gender, clinical stage, food intake (fish consuming), and smoking. The major limitations of this study is the small size of the subjects involved. Therefore, further studies with larger number of CRC blood or tissue samples should be performed to confirm these findings and to study the different mechanisms and pathways involved.

Ethical approval: The Institutional Review Board at King Abdullah Medical City in Holy Capital, Saudi Arabia approved the protocol of this study under reference no. (IRB-002-FR1).

Declaration of interests: The authors declare no conflict of interests.

Author contribution: ABA wrote the manuscript; HAS, HFA, and HMA did the practical work; HMC designed the study; SAR did the statistical analysis; HAA reviewed and critically edited the manuscript.

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