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LncRNA *ES3* Is Upregulated in High-Grade CRC and Its Expression Is Elevated Along with Increasing Tumor Size

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ABSTRACT

Cancer Stem Cell (CSC) is known as a minor percentage of tumor cells inside various malignancies with shared common features with embryonic stem cells such as self-renewal capability and pluripotency. It is believed that not only stemness transcription factors but also pluripotent lncRNAs regulate various aspects of CSCs. It was demonstrated that the embryonic lncRNAs regulate the main features of these cells through interacting with ESC-associated transcription factors. LncRNA *ES3* is a pluripotent lncRNA that was demonstrated to sustain the stemness status of human embryonic stem cells. However, there is little evidence for its contributory role in tumorigenesis. Therefore, in the present study, the possible expression of *ES3* transcript in collected Colorectal Cancer (CRC) tissues and their non-tumor marginal samples were explored by employing the RT-qPCR strategy. Then, we accomplished a survival analysis of *ES3* based on the TCGA database. Inconsistent with metadata, our results revealed that the *ES3* expression markedly increased in CRC tissues rather than non-tumor marginal specimens. Furthermore, we detected that the *ES3* expression meaningfully increased in high-grade and high-stage CRC tissues compared to low-grade and low-stage ones, respectively. Moreover, *ES3* was significantly upregulated in tumor tissues along with increasing tumor size of CRC samples. Our findings, for the first time, demonstrated that lncRNA *ES3* is expressed in CRC and its expression may contribute to the tumorigenesis process and progression of CRC. Hence, lncRNA *ES3* can be regarded as a new cancer biomarker with possible utility in the diagnosis, prognosis, and therapy of colorectal cancer.

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Introduction

Colorectal Cancer (CRC), the third recognized cancer worldwide, is estimated to be responsible for 600,000 cancer-related death per year (Seigal *et al.,* 2020). While researchers try to decipher the regulatory mechanisms of initiation, progression, and invasion of CRC, the molecular biology of CRC is not well understood. Hence, finding the novel and effective factors that cause CRC can be important in prognosis, diagnosis, and targeted therapy of CRC (Chen *et al.*, 2016; Jafari-Oliayi and Asadi, 2018). Tumor-Node-Metastasis (TNM) staging is prevalently the most notable risk factor for CRC and it further has been verified that the tumor size can be considered as a prognostic indicator of CRC (Kornprat *et al.*, 2011; Wang *et al.*, 2016; Dai *et al.*, 2017)

A minor portion of cancer cells, identified as Cancer Stem Cells (CSCs), with common characteristics with stem cells including self-renewal capability and pluripotency, are responsible for tumor reappearance and resistance to chemotherapy (Reya *et al.*, 2001; Singh and Settleman, 2010; Yu *et al.*, 2012). They can generate either from normal stem cells due to dysregulation of Embryonic Stem Cells (ESCs) associated transcription factors or from dedifferentiation and reprogramming of differentiated cells (Macarthur *et al.*, 2009). Thus, it seems that dysregulation of ESC-

associated factors is effective in CSCs originating. Recent reports demonstrated that not only pluripotent coding-transcription regulators including Oct4, Sox2, and Nanog but also stemness lncRNAs can contribute to various regulatory features of CSCs (Jannat Alipoor *et al.*, 2018; Hu *et al.*, 2012; Ng *et al.*, 2012; Ng and Stanton 2013). The stemness lncRNAs such as *MIAT*, *ES1,* and *ES3* regulated the important features of these cells including self-renewality and pluripotency through interacting with embryonic transcription factors (Fatica and Bozzoni, 2014; Ng *et al.*, 2012).

LncRNAs (long non-coding RNAs) are categorized longer than the 200 nucleotides subdivision of non-coding RNAs, which cannot produce functional proteins. Recently, this fraction of non-coding RNA has raised new problems in translational research (Hu *et al*., 2012; Keshavarz and Asadi 2019; Ng *et al.*, 2012; Ng and Stanton 2013; Spizzo *et al.*, 2012). Accumulating reports demonstrated that lncRNAs play a vital role in numerous cellular pathways such as pluripotency, imprinting, cell cycle regulation, proliferation, cellular senescence, apoptosis, and developmental process (Hu *et al.*, 2012; Kim *et al.*, 2015; Ng *et al.*, 2012; Prensner *et al.*, 2013). Recent reports have also shown that lncRNA signatures are promising predictors of survival in patients with CRC (Rezanejad Bardaji *et al.*, 2018; Zhao *et al.*, 2018).

LncRNA *ES3,* as an intergenic lncRNA, is located on chromosome 13q14.3 with 1053 nucleotides length. Recent research claimed that lncRNA *ES3* is highly expressed in ESCs and induced pluripotent stem cells (iPSCs), and have a critical role in maintaining the pluripotency of ESCs through interacting with Sox2, a defined stemness factor in human embryonic stem cells (Hu *et al.*, 2012; Ng *et al.*, 2012). In our previous report, we established that *ES3* transcript is noticeably overexpressed in patients with Her2 positive ductal breast cancer and we determined Her2 regulates the *ES3* expression in breast cancer (Keshavarz *et al.*, 2019). Despite the important role of the transcript in embryonic stem cells and breast cancer cells, its expression in CRC has not been determined yet. Therefore, in this research, we determined the possible expression of lncRNA *ES3* in colorectal cancer by RT-qPCR strategy.

Materials and Methods

Tissue collection

In the current study, cancer tissue samples and corresponding marginal non-tumor specimens from 30 affected patients with CRC and their clinicopathological data were taken from Iran National Tumor Bank (Tehran, Iran). To avoid RNA degradation until its extraction, the specimens were kept in liquid nitrogen. The patients' signed informed consents for participation in the study were taken (Table. 1).

Cell culture

At first, the *ES3* expression in different CRC cell lines was investigated. Therefore, four cancer cell lines derived from colorectal cancer including SW1116, SW480, SW48, and HT-29 were collected from the national cell bank of Iran (Pasteur Institute, Tehran, Iran). Based on the properties of the cell lines and proper conditions for their growth and proliferation, the cells were propagated either in RPMI-1640 or high glucose DMEM. Finally, the cultural mediums were enriched by adding 10 % fetal bovine serum and 100 U/ml penicillin, and 10 µg/ml streptomycin (Gibco, USA), and the proliferating cells finally incubated in the suitable humidity and CO_2 concentration.

RNA extraction and qPCR

The total RNA extraction from cells and tissues was done by solving them in Trizol Reagent (Invitrogen, USA) according to the manufacturer's recommended protocol. The total isolated RNA was quantified by measuring the 260/280-nm ratio with spectrophotometry (Cary 60, USA) and its exact integrity was detected by agarose gel electrophoresis. Purification of the extracted RNA from the co-extracted DNA was performed by its treating with RNase-free DNase I (Fermentas Co., Lithuania). Furthermore, the cDNA was produced by employing an appropriate concentration of extracted RNA, as earlier prepared, as a template for MMLV reverse transcriptase (Fermentas Co., Lithuania) based on the mentioned recommendation in the protocol sheet.

Table 1. The correlation between the expression level of *ES3* and clinicopathologic parameters of CRC tissues.

Clinicopathologic parameters	Number of cases	*ES3* expression		p value
		Low	High	
Age (year)				0.034
≤65	23	5	18	
>65	13	5	8	
Gender				0.975
Male	21	6	15	
Female	15	4	11	
Tumor size (cm)				**0.0010****
≤5	14	6	8	
>5	22	2	20	
Histologic grade				**0.034***
Well or moderate	24	6	18	
Poor	12	2	10	
TNM stage				**0.0401***
I, II	17	4	13	
III, IV	19	4	15	
Lymphatic invasion				0.305
Negative	16	3	13	
Positive	19	4	15	
Vascular invasion				0.305
Negative	16	3	13	
Positive	19	4	15	
Perineural invasion				0.426
Negative	26	4	22	
Positive	9	3	6	

*p < 0.05; **p < 0.01

Briefly, we added dNTP mix (final concentration of 1mM), 20U RNase inhibitor, and random hexamer priming to isolated RNA plus reverse transcriptase solution in a 20µl reaction. The relative expression of the *ES3* gene was determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) rather than β-actin as a housekeeping gene. The precise primers for amplification of *ES3* and β-actin were designed by employing GeneRunner software (version 4.0; Hastings Software. Inc. Hastings, US) and their specificity and correct binding to templates was verified by BLAST (http: //blast. ncbi.nlm.nih.gov/Blast.cgi). The sequence of specific primers is listed as follow:
LncRNA *ES3* (Forward: 5′-AAGACTGACACTGCCCAATCG; Reverse: 5′-GCTGTAGGAAGGTTGTGAGATG); *β-actin* (Forward: 5′-CACTCTTCCAGCCTTCCT TC; Reverse: 5′-AGTCCGCCTAGAATCATT TG). Then, the quantitative polymerase chain reaction (qPCR) was performed employing the SYBR Premix Ex TaqTM II (Takara, Japan) with thermal cycling on ABI Step One Plus real-thermo-cycler in the certain set program for each primer pair. The specificity of PCR products was verified using agarose gels electrophoresis.

Gene expression and survival analysis

We first determined *ES3* expression in different tumor tissues/cell lines especially in CRC and then analyzed the correlation between its expression level and histological classification of CRC. Additionally, the relationship between the expression level of *ES3* and the expression of ESC-associated transcription factors in CRC was analyzed. These expression analyses were performed using the TCGA database in ENCORI starBase v2.0 (Li *et al*., 2014) for RNA interactomes (http://starbase.sysu.edu.cn/) and GENT2 (Park *et al.*, 2019). Furthermore, to identify the relationship between *ES3* expression and survival rate in CRC patients, the TCGA meta-database was used for exploring the patterns of gene expression in normal and tumor

tissues (http://gent2.appex.kr/gent2/). Kaplan–Meier curve was plotted for analyzing the survival rate in patients with CRC according to *ES3* expression level. Based on the TCGA dataset, the CRC patients were categorized into two groups; high risk and low risk determined based on the Cox regression model. The statistical significance difference between the groups was measured using the log-rank test ($p < 0.05$).

Statistical analysis

Real-time PCR reactions were repeated at least three times. The differences between gene expressions in different groups were analyzed using the independent t-test and one-way ANOVA (Analysis of Variance), in the Graph Pad Prism 6.07 software and REST 2009 program. We analyzed the relative level of gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmttigen, 2001). The statistical significance measured was $p < 0.05$.

Results

LncRNA *ES3* is expressed in various cancer tissues, cell lines, and its expression is upregulated in colorectal cancer tissues

At first, the *ES3* expression in a wide range of various types of cancer tissues and cell lines was collected from the TCGA database (Fig. 1A and B). Inconsistent with meta-data, our expression data proved that the *ES3* transcript was expressed in colorectal cancer tissues and cell lines. The results indicated that *ES3* expression level was significantly increased in tumor tissues compared to its expression in adjacent marginal normal tissues ($p = 0.010$, Fig. 1C). Moreover, the expression analysis of *ES3* transcript in colorectal cancer cell lines was done using RT-qPCR, proving that it was expressed in four different colorectal cancer cell lines including SW1116, SW480, SW48, and HT-29 (Fig. 1D).

ES3 transcript is upregulated in high-grade and high-stage colorectal cancer tissues

We further evaluated the relationship between *ES3* expression level and the clinicopathological parameters of CRC tissue samples. Our finding showed that the expression of *ES3* transcript was significantly upregulated in high-grade tumor tissues in comparison to low-grade specimens ($p = 0.050$, Fig. 2A). Furthermore, our expression data indicated that the expression of *ES3* transcript was markedly upregulated in high-stage CRC tissues as well as high-grade tumors ($p = 0.04$, Fig. 2B). In line with the above results, *ES3* expression levels analysis in multiple colorectal cancer samples from the TCGA database showed that it was up-regulated in high-stage colorectal cancer tissues rather than low-stage samples (Fig. 2C). We further observed that patients with high-stage colorectal cancer had significantly shorter overall survival than those with lower stages of CRC because *ES3* had higher expression in high-stage CRC rather than that in low-stage ones (Log-rank $p < 0.001$, Fig. 2D). Moreover, we found that the expression level of *ES3* was dramatically correlated with tumor size and its expression was dramatically elevated in larger tumor tissues compared to smaller ones ($p = 0.010$, Fig. 2E).
Based on the TCGA database in ENCORI starBase v2.0 and GENT2, we detected that the higher expression level of *ES3* was noticeably related to a lower survival rate in patients with colorectal cancer (Log-rank $p = 0.007$, Fig. 3A). Moreover, we found that *ES3* expression was suggestively related to shorter overall survival in poorly differentiated samples of CRC patients (Log-rank $p = 0.002$, Fig. 3B). In addition, we found that there is a noteworthy positive relationship between *ES3* level and NANOG expression in CRC patients based on the analysis of data from the TCGA database in ENCORI starBase v2.0 ($p = 0.005$, Fig. 3C). Additionally, the ROC curve analysis for *ES3* was determined by calculating the part below the (AUC)-ROC curve. This area under the curve was 0.821 leading to an introduction of the transcript as a new diagnostic marker in patients with CRC ($p = 0.003$, Fig. 3D).

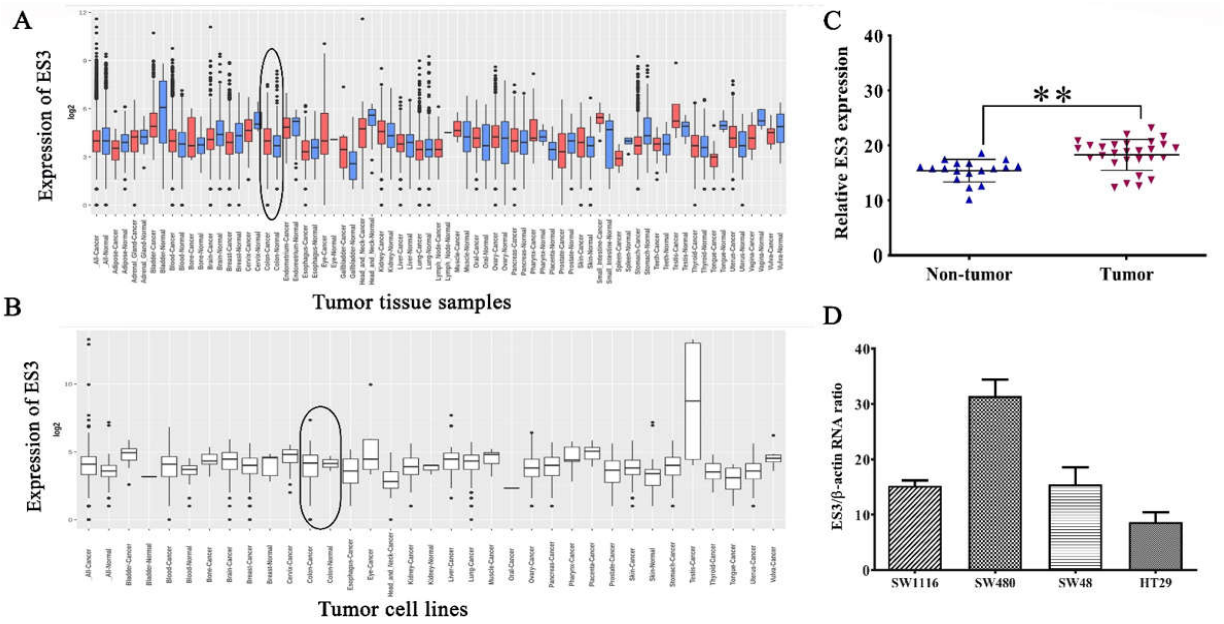

Fig. 1. LncRNA *ES3* is expressed in widely various cancer cells and tissues: A) The expression analysis data which was obtained from TCGA data in GENT2 showed that *ES3* transcript is expressed in widely various cancer tissues. The data suggested that the transcript may play an oncogenic role in numerous cancer types; B) *ES3* expression level in different tumor cell lines which was derived from various cancers in TCGA database; C) RT-qPCR analysis of lncRNA *ES3* expression indicated that its expression is significantly upregulated in CRC tissues rather than that in their corresponding colorectal normal tissues (**p < 0.01); D) The expression analysis data by RT- qPCR revealed that lncRNA *ES3* is expressed in the four immortalized human CRC cell lines, while SW480 cells have the highest *ES3* expression among them, and HT29 cells have the lowest level of *ES3* expression.

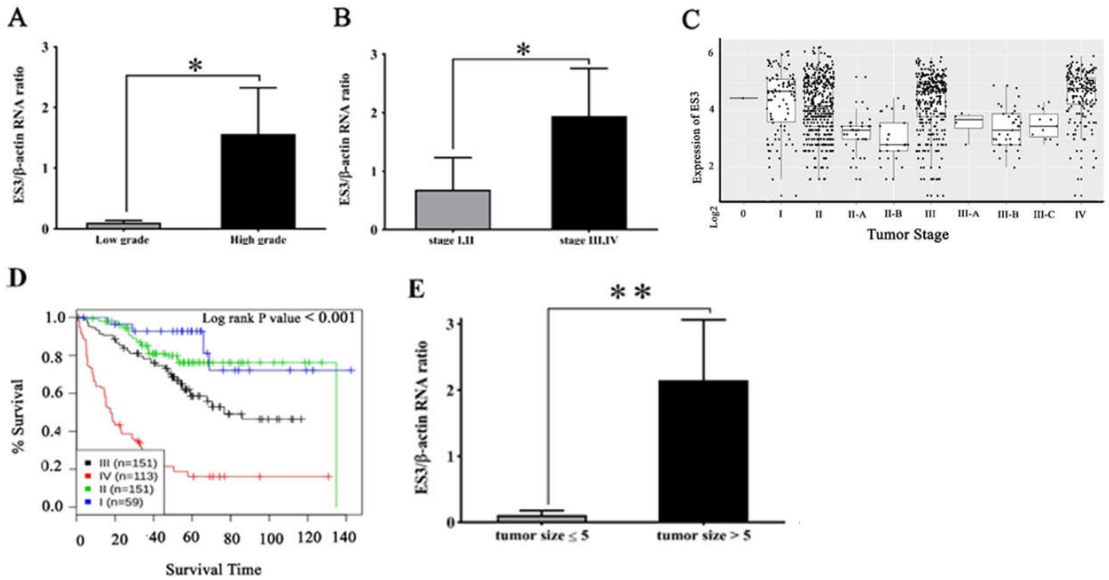

Fig. 2. LncRNA *ES3* is significantly overexpressed in coordinate to the grade/stage increasing in CRC tissues and its expression was dramatically related to tumor size: A) The expression analysis of lncRNA *ES3* in CRC tissues demonstrated that its expression was significantly overexpressed in both high-grade (*p < 0.05); B) and high-stage CRC tissues (*p < 0.05); C) Diagram showed the *ES3* expression level in multi-stages of colorectal cancer on TCGA database.; D) According to the TCGA database, the Kaplan-Meier method was used to analyze the correlation between *ES3* expression in different stages of CRC and the survival rate of patients. This data showed that *ES3* expression was significantly associated with survival rate in higher stages of CRC (*p < 0.001); E) Our RT-qPCR results demonstrated that the expression of lncRNA *ES3* dramatically elevated along with increasing the CRC tumor size.

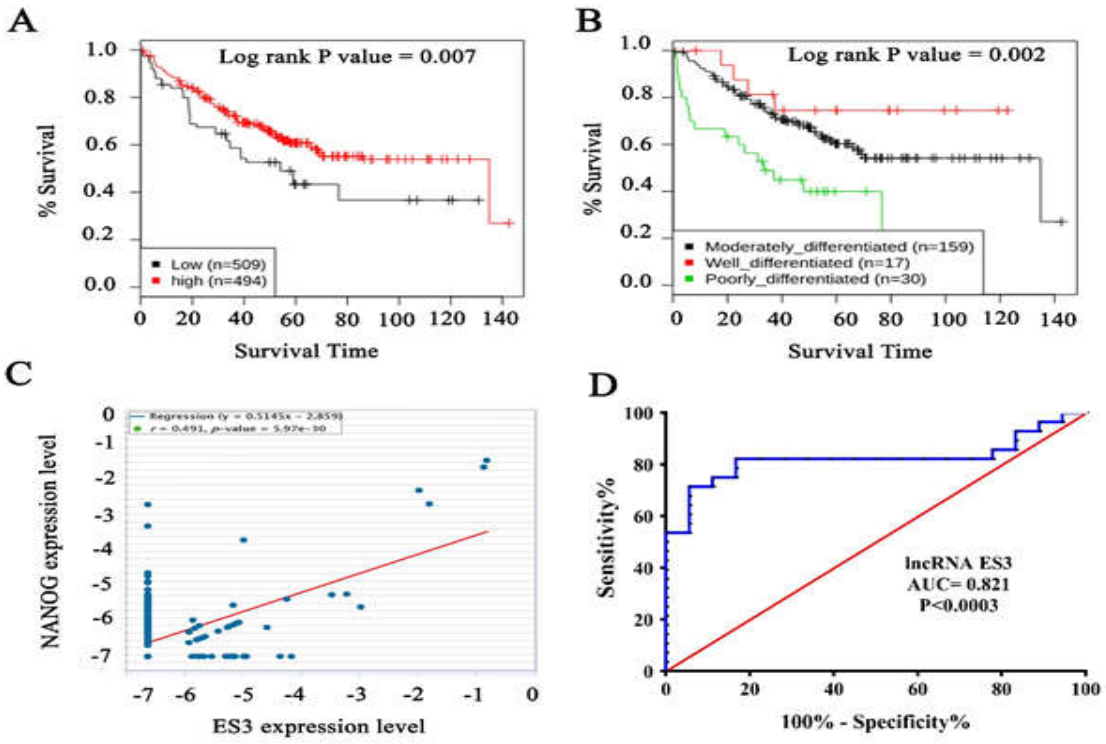

Fig. 3. Analysis of lncRNA *ES3* as a biomarker in CRC tissues based on TCGA database in ENCORI starBase v2.0 and GENT2: A) The *ES3* prognostic value was detected by analyzing the clinical data from the TCGA database and the data demonstrated that it has a significant relationship with overall survival (*p = 0.007); B) The *ES3* prognostic value is significantly related to survival rate in poorly differentiated of CRC (*p = 0.002); C) The significant correlation between the expression level of *ES3* and NANOG in CRC was determined by employing the clinical data from TCGA which was obtained from ENCORI: The Encyclopedia of RNA Interactomes. (*p = 0.005); D) According to the receiver operating characteristic (ROC) curve, the part below the (AUC)–ROC curve introduces lncRNA *ES3* as new a diagnostic biomarker in patients with CRC (*p = 0.003).

Discussion

CRC is known as the third main reason for cancer-related death worldwide. Despite the development of diagnosis and therapy of CRC, tumor recurrence and metastasis are still two important problems of CRC-related death (Garza-Trevino *et al.*, 2015). The cancer stem cell hypothesis claims a portion of tumor cells, which have shared common properties with stem cells account for tumor recurrence and restriction of chemotherapy effects. This theory states that CSCs are originated either from stem cells with dysregulation of stemness factors including ESC-associated transcription factors and pluripotent non-coding RNAs or from reprogramming of differentiated cells (Hu *et al*., 2012; Ng and Stanton, 2013; Keshavarz *et al*., 2019).

In recent years, researchers reported that long non-coding RNAs play a crucial role in the initiation and progression of various types of cancer including CRC (Hu *et al*., 2012; Keshavarz *et al*., 2019; Ng and Stanton, 2013). Linc00458, known as *ES3*, is a pluripotent lncRNA expressed in the nucleus of undifferentiated hESCs and iPSCs indicate that it plays a vital role in pluripotency (Ng *et al.*, 2012). Recent studies established that there is an Oct4- and NANOG-binding site vicinity lncRNA *ES3* transcription start site. The neighboring binding sites directly regulate the expression of lncRNA *ES3* and knocking-down of Oct4 and NANOG leads to *ES3* downregulation (Ng *et al.*, 2012). Previous reports showed that the stemness coding factors such as NANOG can induce a stem-like state and be involved in the progression of CRC (Zhang *et al.*, 2013; Gawlik-Rzemieniewska and Bednarek 2016). NANOG expression is significantly correlated with high expression of E-cadherin, larger tumor sizes, and vascular invasion (Gawlik-Rzemieniewska and Bednarek 2016).

Our previous report established that lncRNA *ES3* was expressed in both tumor and stem cells (Keshavarz *et al.,* 2019). Thus, we suggested that its dysregulation in cancer cells, based on CSCs theory, may involve tumorigenesis in different cancer types. In line with the data, in current research, we explored the expression of *ES3* in a wide range of various tumor tissues and cell lines, derived from different cancer types based on the TCGA database and we observed that *ES3* transcript is expressed in numerous cancer cells. The results supposed that lncRNA *ES3* may act as a novel oncogene in different cancer types. In the subsequent phase of the current research, we studied the possible expression of *ES3* in CRC tissues and marginal normal samples. Consistently, we found that the expression of *ES3* was upregulated in CRC tissues compared with marginal normal tissues. Thus, we suggested the transcript may be involved in CRC tumorigenesis and it may act as an onco-lncRNA in CRC. Furthermore, we found that overexpression of this lncRNA is meaningfully related to the pathological grade, stage, and tumor size in patients with CRC. Our data revealed that there is a significant association between the tumor grades and *ES3* expression. On the other hand, *ES3* expression was inversely correlated with differentiation of CRC tissues, so that its expression markedly elevated along with increasing tumor grades. Our data suggested that *ES3* transcript may contribute to tumor progression of CRC and it can be effective as a prognostic and diagnostic factor of poorly differentiated CRC. Furthermore, we found that the expression level of lncRNA *ES3* is dramatically correlated with tumor size and its expression is significantly upregulated along with increasing the size of tumor tissues. It has been established that the tumor size is correlated with tumor invasion, progression-free, and cancer-specific survival (Dai *et al.*, 2017). In this way, we analyzed the expression of the *ES3* in colorectal adenocarcinoma from data derived from the TCGA database in ENCORI starBase v2.0 and GENT2. We found that *ES3* is associated with the overall survival of patients with colorectal cancer. We use survival rate in overall, grading and staging system for further identifying the *ES3* signature. *ES3* was suggestively associated with overall survival, high-stage, and poorly differentiated CRC sample and it can be considered as a notable probable biomarker for CRC prognosis. Moreover, TCGA database analysis showed that there is a noteworthy positive correlation between *ES3* and NANOG expression that supports *ES3* relevance with NANOG as an effective factor in cellular proliferation, survival rate, and tumor size in CRC. Previous report claimed that the *ES3* expression was regulated by Nanog (Ng et al., 2012) and based on the data obtained from TCGA, we suggested that *ES3* and NANOG may contribute to CRC tumorigenesis by acting in the same regulatory pathway. Hence, we supposed that *ES3* transcript may have a crucial role in tumor progression and invasion of poorly differentiated CRC.

In conclusion, we provided the initial sign that lncRNA *ES3* was significantly up-regulated in colorectal cancer tissues compared to marginal normal tissues. Statistical analysis revealed that *ES3* expression in CRC patients is associated with tumor grade, size, stage, lymph node metastasis, vascular and perineural invasion. This result indicates that lncRNA *ES3* could serve as a predictor of advanced CRC. In summary, for the first time, our study showed that *ES3* expression in colorectal cancer was associated with tumor grade, and invasion, introducing *ES3* transcript as an oncogene in the progression and metastasis of CRC.

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