

Expression of long non-coding RNA (lncRNA) H19 in immunodeficient mice induced with human colon cancer cells

Q. HU¹, Y.-B. WANG², P. ZENG², G.-Q. YAN¹, L. XIN¹, X.-Y. HU¹

¹Gastrointestinal Surgery Department, The Second Affiliated Hospital of Nanchang University, Nanchang, China

²Emergency Department, The Second Affiliated Hospital of Nanchang University, Nanchang, China

Qian Hu, Yi-Bing Wang contributed equally in this work

Abstract. – OBJECTIVE: To study the expression of long non-coding RNA (lncRNA) H19 in immunodeficient mice induced with human colon cancer cells. Long non-coding RNAs (lncRNA) have a vital and regulatory role, in almost all cancers including colon cancer. In the present study, the prominent regulatory role of lncH19 RNA in immunodeficient mice induced with human colon cancer cells was identified. Interestingly, the lncH19 RNA was up-regulated in human colon cancer cells induced immunodeficient mice colon cancer samples.

PATIENTS AND METHODS: The expression profile of the lnc H19 RNA in the colon cancer tissues were identified by RT-PCR and the further the data was confirmed and validated by Northern blot analysis.

RESULTS: Based on the data, it was identified that lnc H19 RNA was increased significantly in immunodeficient mice induced with human colon cancer cells, when compared with controls. The data was further confirmed and validated by Northern blot analysis.

CONCLUSIONS: The obtained data future potential application in colon cancer diagnosis. At the same time, lncH19 RNA may serve as a new target for anti-tumor therapy in colon cancer. Also, the obtained data is the important clue to find out the key roles of lncH19 RNA-miRNA functional network in colon cancer.

Key Words:

Colon cancer, lnc H19 RNA, NOD/SCID mice, RT-PCR.

Introduction

Colon cancer is the second most common cancer causing death in human population^{1,2}. Novel in-depth studies are essential to diagno-

se the colon cancer in earlier stages, which is the key to fighting against and demolish the disease. One such potential candidate is long non-coding RNAs, which have a vital regulatory role in most cancers including breast cancer. The over-expression of STMN1 in colon cancer is independently linked with the development of the disease³. Recently the trace element Selenium (Se) is used in the clinical treatment of colorectal cancers⁴. Notably, the human genome has nearly greater than 98% of Junk DNAs, which is commonly named as non-coding RNAs (ncRNAs), specifically grouped into short ncRNAs and long ncRNAs (lncRNAs)⁵. Though it is junk DNA, their function related to the cellular mechanism and relevance to the diseases is still not clear⁶. The role of short ncRNAs has been determined, which has specific cellular mechanisms such as modifying the alternative splicing process, remodeling the chromatin and RNA metabolism respectively⁷⁻¹⁰. But, lncRNAs are structurally and functionally different from short ncRNAs¹¹. At the same time, it is identified that the following long non-coding RNAs (lncRNAs) such as MALAT-1, H19, and HO-TAIR have an important role in the aspect of carcinogenesis or cancer growth^{6,12-16}. lnc H19 RNA levels are up-regulated during pro-tumorigenic properties as well as after exposure to hypoxia¹⁷. In contrast, lnc H19 RNA has been considered as an oncogenic lncRNA in hepatocellular and bladder carcinoma¹⁸⁻²². Based upon the importance of lnc H19 RNA in cancers, the present work was designed to identify the expression pattern of lnc H19 RNA and its role in colon cancer.

Patients and Methods

Colon Cancer Sample Collection

Colon cancer samples were collected from the hospital with prior written consent from the patients. The research project was accepted by the ethical committee of the hospital as well as the institution. The age of the patients was ranged from 22 to 45 years. Immediately after tumor sample collection, the samples were subjected to mechanical dissociation and incubated with Collagenase Type IV (Invitrogen, Carlsbad, CA, USA). The samples were injected into the NOD/SCID mice for the initiation of the colon cancer with human colon cancer cells. All biopsy tumor specimens was crosschecked histologically to have >90% of tumor cells.

NOD/SCID Mice and Transplantation of Human Colon Cancer Cells

NOD (NOD/SCID) mice were kept under defined conditions in the animal house with the approval by the Animal Care Committee of the Institution. Colon cancer cells were suspended in the media Matrigel (BD Biosciences, San Diego, CA, USA) at the ratio of 1:1 mixture and injected under the renal capsule of anesthetized mice (8 weeks of age) that were sublethally irradiated (350 centigray). All mice were killed at the first sign of colon cancer, which is between 15 and 21 weeks of post-transplantation.

RNA Isolation from Samples

TRIzol method was used to isolate total RNA from colon cancer developed mice and control samples as per the standard manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). In brief, the colon cancer samples were lysed with the help of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and quality of the RNA were measured by NanoDrop (NanoDrop 2000, Wilmington, DE, USA). Also, the integrity was crosschecked by the general protocol of Agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA). The isolated RNA was used throughout the experiments specifically for RT-PCR and Northern blot analysis.

RT-PCR Analysis

Total RNA was isolated from the tumor specimen samples and stored at -70°C using standard manufacturer protocol (RNeasy Mini kit) (Qiagen, Manchester, UK) and treated with DNaseI (Invitrogen, Carlsbad, CA, USA). The quality and quantity of the RNA were validated

by standard procedures. Reverse transcription was performed using 0.5 µg total RNA as a template and PCR was performed on 1/20 of the RT product using the following primers; H19 F, 5'-CTGTAACCGGCGCCAGAA-3' and R, 5'-TGCATGGGAGAGCCCAGA-3'; and; β-actin F, 5'-TGGAGAGGCCTGCCAAGTA-3' and R, 5'-AAGAGTGGGAGTTGCTGTTG-3'. The cycles were run under standard condition as per the protocol and the PCR products were resolved in 2% Agarose gel (Sigma-Aldrich, St. Louis, MO, USA) having ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA), observed and documented using gel documentation unit (Bio-Rad, Hercules, CA, USA).

Northern Blot Analysis

RNA samples were prepared from the colon cancer mice and also from control tissues samples and resolved in the 1.2% Agarose gel (Sigma-Aldrich, St. Louis, MO, USA). The resolved RNA samples were transferred to nylon membrane, and hybridized with lnc H19 RNA specific biotin-labeled DNA probes (Thermo Scientific, Waltham, MA, USA). HRP conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) were (1:10000) used and developed with DAB/H₂O₂ (Amresco, Texas, TX, USA) for a brown colored product on the membranes.

RNA Immunoprecipitation

RNA immunoprecipitation was performed using Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). The p53 antibody ab2433 (Abcam, Cambridge, MA, USA) was used for the RNA immunoprecipitation. Primers 5'-GGGTCTGTTTCTTTACTTCCTCCAC-3' and 5'-GATGTTGGGCTGATGAGGTCTGG-3' were used to identify the H19 expression.

Results

To study the role and importance of lnc H19 RNA in colon cancer following experiments were performed, human colon cancer samples were collected from the hospital through an institutional review board approved committee. Also, the Ethical Committee of the hospital approved the project. Immediately after collection, immunodeficient mice were taken and induced with human colon cancer cells. All biopsy tumor specimens were crosschecked histologically to have >90% of tumor cells.

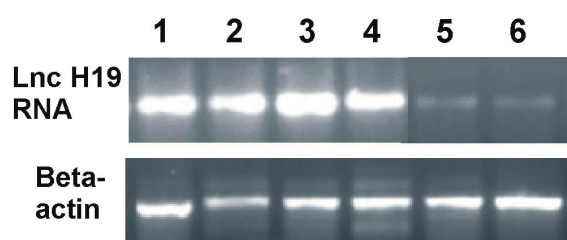


Figure 1. RT-PCR Results. Total RNA was extracted from immunodeficient mice induced with human colon cancer cells and control mice followed by RT-PCR with Lnc H19 RNA specific primer, along with beta-actin as control; Lane 1 to 4 – Colon cancer samples; Lane 5 to 6 – Control samples.

RT-PCR Analysis

Total RNA was isolated from the tumor specimen samples; RT-PCR was performed using the standard protocol as described earlier, along with controls. The results of RT-PCR were shown in Figure 1. It was identified that over-expression of Lnc H19 RNA was observed in all colon cancer samples except control. The results were repeated thrice and same results were obtained. Based upon the preliminary data it was confirmed that Lnc H19 RNA was over expressed in colon cancer immunodeficient mice induced with human colon cancer cells and obviously, it was also identified that Lnc H19 RNA was not overexpressed in the control mice. To validate the results, northern blot analysis was performed.

Northern Blot Analysis

To confirm the preliminary data that Lnc H19 RNA was overexpressed in immunodeficient mice induced with human colon cancer cells, northern blot analysis was performed. Total RNA was isolated from colon cancer tumor specimen samples and control; Northern blot analysis was performed using a standard protocol. The data was shown in Figure 2. It was identified that the over-expression pattern of Lnc H19 RNA was observed in the all the colon cancer specimen samples, as similar with the data obtained from RT-PCR results. The experiment was repeated thrice and the same results were obtained. Based upon the data it was confirmed and validated that the Lnc H19 RNA was overexpressed in immunodeficient mice induced with human colon cancer cells when compared with control. Similarly, it was also identified that Lnc H19 RNA expression was not observed in the control mice.

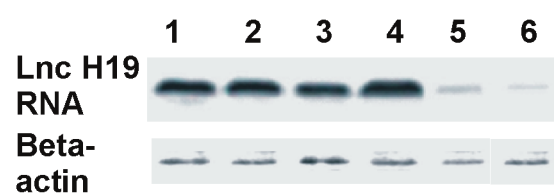


Figure 2. Northern blot analysis. Colon cancer samples and control samples were resolved in Agarose gel and hybridized with biotin-conjugated Lnc H19 DNA specific probe. Lane 1 to 4 – Colon cancer samples; Lane 5 to 6 – Control samples.

Lnc H19 RNA and Suppressed p53 Activation

To identify the mechanistic role of Lnc H19 RNA in colon cancer growth, following experiments were performed from nuclear extracts of colon cancer samples. For RNA immunoprecipitation experiments, an antibody against p53 was used to identify the link between Lnc H19 RNA and p53. The results were shown in Figure 3. Based upon the data it was identified that notable enrichment of Lnc H19 RNA was observed using the p53 antibody, when compared with IgG control antibody.

Discussion

Colon cancer is the second most common cancer¹ causing death in the human population. Very limited promoting agents for colon cancer have been previously reported, notably, MACC1 promotes the growth and metastasis of colon cancer cells²³. Molecular analysis in the context of junk DNA molecules which codes for long non-coding RNAs and its link with cancer is not studied well. Long non-coding RNAs (lncRNAs) such as MALAT-1, H19 and HOTAIR have an important role in carcinogenesis or cancer growth^{6,12-16}. With this context, present experiment was performed. Colon cancer samples were collected from the

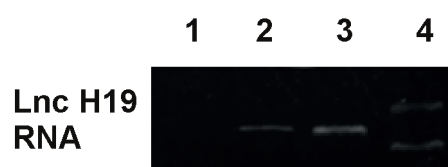


Figure 3. RNA immunoprecipitation with p53 antibody. Nuclear extract of tumor specimen samples were subjected to RNA immunoprecipitation assay with p53 antibody; Lane 1 – Ig G control, Lane 2 – RNA immunoprecipitation with p53 antibody, Lane 3 – Total RNA and Lane 4 – Marker.

patients as described in the Patients and Methods. The tumor specimen samples were analyzed histologically and confirmed to contain >90% tumor cells.

To identify the expression profile of lnc H19 RNA in colon cancer samples as well as in control, total RNA was isolated and RT-PCR was performed using lnc H19 RNA specific primer. The data was shown in Figure 1, which illustrates that lnc H19 RNA was overexpressed in colon cancer samples when compared with that of control. The expression profiles of lnc H19 RNA in control samples were normal. The preliminary data suggest that lnc H19 RNA was over expressed in immunodeficient mice induced with human colon cancer cells. The obtained results coincided with the previous reports^{6,12-16}, which showed that long non-coding RNAs (lncRNAs) such as MALAT-1, H19 and HOTAIR have an important role in carcinogenesis or cancer growth. Also, it has been well documented that lnc H19 RNA is an oncogenic long noncoding RNA in hepatocellular and bladder carcinoma¹⁸⁻²², but in the present study, lnc H19 RNA was markedly elevated in immunodeficient mice induced with human colon cancer cells. Thus, it was clearly identified that, lnc H19 RNA is an oncogenic long noncoding RNA, which was overexpressed in colon cancer.

To validate the data further, Northern blot analysis was performed. The data (Figure 2) shows that, lnc H19 RNA was elevated in the colon cancer samples, which was not elevated in the control samples. The Northern blot data in combination with RT-PCR data confirmed and validated that, lnc H19 RNA was over expressed in immunodeficient mice induced with human colon cancer cells. It is interesting that the junk DNA molecules is important and perform various signaling pathways, especially in cancers.

In order to identify the link of junk DNA molecules in colon cancer, RNA immune precipitation assay was performed. The findings (Figure 3) confirm that lnc H19 RNA has a link with p53. Based upon the data (Figures 1, 2), it was confirmed that prominent enhancement was noted in lnc H19 RNA with p53. Hence it was concluded that the junk DNA molecules have a valuable role in colon cancer.

Conclusions

The obtained results will be used as a potential application in colon cancer diagnosis, as

well as lncH19 RNA may serve as a potential new target for anti-tumor therapy in colon cancer. Also, the obtained data is the important clue to find out the key roles of lncH19 RNA-miRNA functional network in colon cancer.

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Conflicts of interest

The authors declare no conflicts of interest.

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