Short Report

Long non-coding RNA NEAT1 is a transcriptional target of p53 and modulates p53-induced transactivation and tumor-suppressor function

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p53 is one of the most important tumor suppressor genes, and the direct transcriptional targets of p53 must be explored to elucidate its functional mechanisms. Thus far, the p53 targets that have been primarily studied are protein-coding genes. Our previous study revealed that several long non-coding RNAs (lncRNAs) are direct transcriptional targets of p53, and knockdown of specific lncRNAs modulates p53-induced apoptosis. In this study, analysis of next-generation chromatin immunoprecipitation-sequencing (ChIP-seq) data for p53 revealed that the lncRNA NEAT1 is a direct transcriptional target of p53. The suppression of NEAT1 induction by p53 attenuates the inhibitory effect of p53 on cancer cell growth and also modulates gene transactivation, including that of many lncRNAs. Furthermore, low expression of NEAT1 is related to poor prognosis in several cancers. These results indicate that the induction of NEAT1 expression contributes to the tumor-suppressor function of p53 and suggest that p53 and NEAT1 constitute a transcriptional network contributing to various biological functions and tumor suppression.

Introduction

p53 is one of the most important known tumor suppressor genes. In approximately half of all human cancers, p53 is inactivated as a direct result of mutations in the p53 gene.1 Furthermore, mutation or deletion of p53 is related to a poor prognosis and resistance to chemotherapy and radiation.2 The p53 protein is activated by various cell stresses, such as DNA damage, oncogene activation, spindle damage and hypoxia. Activated p53 transactivates several target genes, many of which are involved in DNA repair, cell cycle arrest and apoptosis.3 Furthermore, p53 transactivates not only coding genes but also non-coding RNA genes, including miRNAs, which are major mediators of gene suppression induced by p53.4 A recent study revealed that long non-coding RNAs (lncRNAs) play important roles in various biological and pathological processes such as development, differentiation, stemness and carcinogenesis.5,6 In a previous study, through a combination of chromatin immunoprecipitation-sequencing (ChIP-seq) and in silico analyses, we found several lncRNAs that are upregulated by the p53 family. Additionally, knockdown of specific lncRNAs modulated p53-induced apoptosis and promoted the transcription of a gene cluster.7 Our results suggest that p53 family members and lncRNAs constitute a complex transcriptional network involved in various biological functions and tumor suppression.

In this study, we found that the lncRNA NEAT1 is a direct transcriptional target of p53. Knockdown of NEAT1 affected gene transactivation of p53 and enhanced cancer cell growth. Furthermore, low expression of NEAT1 was related to a poor prognosis in several cancers. These results indicate that the induction of NEAT1 expression contributes to the tumor-suppressor function of p53.

Materials and Methods

Cell culture

Human osteosarcoma SaOS2 and U2OS cells, breast cancer MCF7 cells, and lung cancer A549 and H1299 cells were purchased from the American Type Culture Collection and the Japan Collection of Research Bioresources. Colon cancer HCT116 (p53+/+) and the derivative HCT116 (p53/−/−)
cell line were kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD). The construction, purification, and infection of replication-deficient recombinant adenoviruses containing FLAG-tagged p53 (Ad-p53) and TAp73β (Ad-p73β) or the bacterial lacZ gene (Ad-LacZ) were performed as previously described.8–10 Chromatin immunoprecipitation (ChIP) H1299 cells were infected with Ad-p53 and LacZ at a multiplicity of infection (MOI) of 25. At 24 h after infection, these cells were subjected to ChIP with an anti-FLAG antibody or normal mouse IgG as a control using a ChIP Assay Kit (Upstate) according to the manufacturer’s protocol. ChIP-seq data were deposited into the DDBJ sequence read archive (DRA, accession number: DRA000614) as reported in a previous study.7 The ChIP-seq data from U2OS cells were obtained from SRA: SRX275504. The primer sequences for PCR were CCGCACTGCGGGAATCTT and CCTGTGCTTC. Luciferase reporter assay NEAT1 siRNA (sense, 5’-GCAGAAACCTGGGGCTTGCCG-3’; antisense, 5’-GGGAAGUUUCUAAGCAGGCUUCUCACUUU-3’) and control siRNA (sense, 5’-AAACAGGUUCUCAAGAGAGCUGACUG-3’; antisense, 5’-GGAAAGUUUCUAAGCAGGCUUCUCACUUU-3’) were synthesized by Sigma based on sequences reported in a previous study.11 Cell growth assay U2OS and A549 cells were transfected with siRNA-NEAT1 or control siRNA using Lipofectamine RNAiMAX (Thermo Scientific). After treatment with nutlin-3a or adriamycin, cell growth was quantified using a Cell Titer-Glo 2.0 assay (Promega) according to the manufacturer’s protocol, as previously described.12 RNA sequencing analysis RNA sequencing (RNA-seq) was performed using the HiSeq2500 (Illumina) platform according to the manufacturer’s protocol. The acquired sequence reads were aligned to the human genome sequence (hg19) using TopHat2.13 The expression of each gene was quantified using Cuffquant and normalized with Cuffnorm.14 The expression data were deposited into the NCBI Gene Expression Omnibus (GEO, accession number: GSE85831). Genes with a more than two-fold change in expression in U2OS cells transfected with negative control siRNA and treated with nutlin-3a (10 μM) compared with control cells not treated with nutlin-3a were selected for further analysis. The expression levels were converted to Z-scores and were subjected to hierarchical clustering based on the average Euclidean distance using gplots (R package). Genes included in the specific gene clusters were
analyzed using DAVID (The Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov).15

Analysis of gene expression datasets
In two gene expression datasets from human cancers that included information on survival (GEO: GSE17537, GSE31210, and GSE3494), the patients were divided into high and low NEAT1 expression groups. A survival curve was constructed using the Kaplan–Meier method in survfit (R package). p-Values were calculated using the log-rank test in survdiff (R package).

Results
Binding of p53 to the NEAT1 promoter and transcriptional activity
We analyzed next-generation ChIP-seq data from p53-overexpressing H1299 cells obtained in our previous study7 and public data from U2OS cells treated with nutlin-3a, a small-molecule that activates endogenous p53. Analyzing both datasets, we identified a ChIP-seq peak in the promoter region of the NEAT1 gene (Fig. 1A), and the sequence of the peak corresponded to a consensus p53 motif [RRRCWWGYYY RRRCWWGYYY] (RE in Fig. 1A). Furthermore, we confirmed the interaction between p53 and NEAT1-RE with ChIP-PCR (Fig. 1B). To investigate whether the NEAT1 gene is a direct target of transcriptional activation by p53, we determined whether the NEAT1-RE sequences confer transcriptional activity in a p53-dependent manner. We also performed a reporter assay using luciferase vectors that included NEAT1-RE sequences. H1299 and SaOS2 cells (both p53 null) were transiently cotransfected with the luciferase vector together with a p53-expression plasmid.
significantly increased the luciferase activity in both cell lines, whereas the activity was not increased in cells transfected with a mutated RE (Fig. 1C). These results indicate that p53 directly transactivates the NEAT1 gene by binding to p53 response elements. In addition, the RE sequences and their relative position to the transcription start site (TSS) are well-conserved among multiple mammalian species (Table S1), indicating the significant function of p53 in NEAT1 transactivation.

**Induction of NEAT1 expression by p53**

Human NEAT1 has two isoforms: NEAT1_1 and NEAT1_2. NEAT1_1 is 3.7 kb long, unspliced and poly-adenylated. NEAT1_2 is 23 kb long and is also unspliced. NEAT1_1 is expressed widely in most human tissues, whereas the expression of NEAT1_2 is not abundant. To confirm the transactivation of NEAT1 by p53, U2OS osteosarcoma cells, A549 lung cancer cells, and MCF7 breast cancer cells (all containing wild-type p53) were treated with nutlin-3a. In all of the tested cell lines, nutlin-3a treatment increased the expression of total NEAT1 and NEAT1_2 as well as p21, a canonical p53 target gene (Fig. 2A). Similar results were obtained with another internal control (Fig. S1). Interestingly, p21 and NEAT1 were maximally induced at different concentrations of nutlin-3a (10 and 5 μM, respectively). This result indicates that weak activation of p53 is preferable for the induction of NEAT1 compared with p21. Furthermore, NEAT1 expression was significantly increased by overexpression of p53 but not p73β, a member of the p53 family, in H1299 cells (Fig. 2B). In addition, we quantified the

![Graphs showing NEAT1 expression and p21 induction](image-url)
expression of NEAT1 in isogenic colon cancer cells (HCT116 (p53+/+) and HCT116 (p53−/−) cells) and found that NEAT1_2 was significantly upregulated following adriamycin treatment in HCT116 (p53+/+) cells but not increased at all in HCT116 (p53−/−) cells (Fig. 2C). Curiously, total NEAT1 expression in HCT116 (p53+/+) cells was not affected by adriamycin treatment. In contrast, the treatment decreased NEAT1 expression in HCT116 (p53−/−) cells. Thus, it should be considered that p53-independent biological effects of adriamycin caused NEAT1 levels to decrease in both cell lines but that the increase in total NEAT1 expression resulting from p53 activation by adriamycin in the p53+/+ cells was counteracted by the p53-independent effects. Together, these results demonstrate that NEAT1 is upregulated in a p53-dependent manner.

**Effect of NEAT1 induction on the tumor-suppressor function of p53**

To evaluate the biological effect of NEAT1 downregulation on the tumor-suppressor function of p53, we transfected U2OS and A549 cells with siRNA-NEAT1 or negative control siRNA. Knockdown of NEAT1 was confirmed by RT-qPCR (Fig. 3A). We evaluated cell growth after treatment with nutlin-3a or adriamycin, both of which activate p53. Interestingly, treatment with either agent inhibited cancer cell growth, whereas NEAT1 knockdown partially, but significantly, treatment with either agent inhibited cancer cell growth, whereas NEAT1 knockdown partially, but significantly, restored the cell growth of both cell lines after each of the treatments (Fig. 3B).

Next, to explore the effect of NEAT1 on gene transactivation induced by p53, we evaluated changes in gene expression using RNA-seq in NEAT1 knockdown or control U2OS cells. We selected 2,595 genes that demonstrated more than two-fold increases in expression in control cells treated with nutlin-3a or adriamycin, both of which activate p53. Interestingly, treatment with either agent inhibited cancer cell growth, whereas NEAT1 knockdown partially, but significantly, restored the cell growth of both cell lines after each of the treatments (Fig. 3B).

**Correlation between NEAT1 expression and prognosis in cancers**

To examine whether NEAT1 expression affects cancer prognosis, we surveyed gene expression datasets from human cancers that included survival information, and survival curves were constructed using the Kaplan–Meier method. We selected datasets obtained using an Affymetrix U133 microarray. Paraspeckles have important roles in controlling gene expression through the retention of RNA containing double-stranded RNA regions. During the preparation of this manuscript, we learned that Adriaens et al. also identified NEAT1 as a transcriptional target of p53, and they reported the formation of paraspeckles by p53 activation. Therefore, the formation of paraspeckles may have played a role in our findings that NEAT1 modulates p53-induced transactivation. Several recent studies have indicated that NEAT1_2, but not NEAT1_1, is essential for the formation of paraspeckles. Furthermore, low expression of NEAT1_2 as well as total NEAT1, is correlated with poor survival (Fig. 3D). These facts suggest that NEAT1_2 is functionally important as a p53 target.

**Discussion**

In this study, we showed that NEAT1 is a direct transcriptional target of p53 (Figs. 1 and 2), and the suppression of NEAT1 induction by p53 attenuated the inhibitory effect of p53 on cancer cell growth (Fig. 3B). In addition, we found gene clusters that were increased by p53 only in control cells and not in NEAT1 knockdown cells (Fig. 3C), and these clusters included many lncRNAs. This result indicates that p53-induced NEAT1 expression is required for the induction of the other p53-targeted genes and lncRNAs included in the clusters. Although we performed gene ontology analysis of genes included in the clusters, no functional enrichment was observed (data not shown). However, this type of analysis is based on known functions of coding genes, and functions of lncRNAs included in the clusters are still unknown. We speculate that these lncRNAs play important roles in the tumor-suppressive function of p53. Thus, our results suggest that p53 and NEAT1 constitute a transcriptional network that contributes to various biological functions and tumor suppression.

Recent studies have revealed that NEAT1 is an essential component of paraspeckles. Paraspeckles, a recently identified and novel type of nuclear compartment, have been found in the inter-chromosomal regions of all tested cell types and are therefore thought to have an important role in nuclear function. Paraspeckles have important roles in controlling gene expression through the retention of RNA containing double-stranded RNA regions. During the preparation of this manuscript, we learned that Adriaens et al. also identified NEAT1 as a transcriptional target of p53, and they reported the formation of paraspeckles by p53 activation. Therefore, the formation of paraspeckles may have played a role in our findings that NEAT1 modulates p53-induced transactivation. Several recent studies have indicated that NEAT1_2, but not NEAT1_1, is essential for the formation of paraspeckles. Furthermore, low expression of NEAT1_2 as well as total NEAT1, is correlated with poor survival (Fig. 3D). These facts suggest that NEAT1_2 is functionally important as a p53 target.

Based on an analysis of a cohort dataset of high-grade ovarian carcinoma, Adriaens et al. found that expression of NEAT1_2 predicts the response of ovarian cancer to chemotherapy. At first glance, their results in relation to ovarian cancer appear to be incompatible with our data regarding colon, lung and breast cancers; NEAT1 loss in some cancers suggests that it is a progression-free survival (PFS) marker, whereas it is associated with poor prognosis in others. However, this discordance may reflect different aspects of NEAT1 functions in each cancer type—that is, whether chemotherapy
Figure 3. Effect of p53-induced NEAT1 on cancer cell growth, gene transcription and cancer prognosis. (A) U2OS cells were transfected with siRNA-NEAT1 or negative control siRNA. Twenty-four hours after transfection, these cells were treated with or without nutlin-3a (10 μM). Twenty-four hours after treatment, mRNA was extracted and analyzed by RT-qPCR with primers specific for the siRNA target site. Error bars indicate the SD. (B) U2OS cells and A549 cells were transfected with siRNA-NEAT1 or negative control siRNA. Twenty-four hours after the transfection, the cells were treated with nutlin-3a (10 μM) for 48 h or adriamycin (0.5 μg/mL) for 24 h. After the treatment, cell growth was quantified. Error bars indicate the SE. The asterisk indicates a p value < 0.05 as determined by a t-test. (C) U2OS cells were transfected with siRNA-NEAT1 or negative control siRNA. Twenty-four hours after transfection, these cells were treated with or without nutlin-3a (10 μM). Twenty-four hours after treatment, mRNA was extracted and analyzed by RNA-seq. Genes with a greater than twofold increase in expression in cells treated with nutlin-3a compared with untreated cells were selected, and hierarchical clustering analysis was performed. The red and green colors in the heat map indicate positive and negative Z-scores, respectively. Brackets A and B indicate the gene clusters that were increased by nutlin-3a treatment in control cells, but not in NEAT1-knockdown cells. (D) Correlation between NEAT1 expression and prognosis among cancer patients. For the three indicated datasets, the correlation between total NEAT1 or NEAT1_2 expression (Affymetrix microarray probe: 224566_at and 234989_at) and survival was analyzed and plotted using the Kaplan-Meier method. The survival rates for patients with high and low NEAT1 expression are plotted as red and blue lines, respectively. The number of patients in each group is shown in parentheses. p values were calculated using a log-rank test.
responsiveness or cancer cell proliferation is predominantly affected by NEAT1.

Our analysis indicated that low expression of NEAT1 was associated with poor prognosis in colon, lung, and breast cancers (Fig. 3D), whereas the opposite trends was observed in brain tumors (Fig. S2, top and middle). Interestingly, high expression of p21, one of the most important p53 target genes, was also significantly associated with poor prognosis in brain tumors (Fig. S2, bottom). We speculate that these differences may be caused by a lower rate of p53 mutation in the brain tumors represented in the datasets. As tumors that have wild-type p53 become more aggressive, oncological stresses are also increased, likely resulting in activation of intact p53 and induction of p53 target genes.

References