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Identification of short-lived long non-coding RNAs as surrogate indicators for chemical stress response

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ABSTRACT

Abiotic and biotic stressors in human cells are often a result of sudden and/or frequent changes in environmental factors. The molecular response to stress involves elaborate modulation of gene expression and is of homeostatic, ecological, and evolutionary importance. Although attention has primarily focused on signaling pathways and protein networks, long non-coding RNAs (ncRNAs) are increasingly involved in the molecular mechanisms associated with responses to cellular stresses. We identified six novel short-lived long ncRNAs (MIR22HG, GABPB-AS1, LINC00152, IDI2-AS1, SNHG15, and FLJ33630) that responded to chemical stressors (cisplatin, cycloheximide, and mercury (II) oxide) in HeLa Tet-off cells. Our results indicate that short-lived long ncRNAs respond to general and specific chemical stressors. The expression levels of the short-lived long ncRNAs were elevated because of prolonged decay rates in response to chemical stressors and interruption of RNA degradation pathways. We propose that these long ncRNAs have the potential to be surrogate indicators of cellular stress responses.

1. Introduction

At all stages during an organism's life cycle, cells are subjected to sudden changes or frequent fluctuations in their environment and physiology. Such changes can damage macromolecules that need to be replenished, including DNA, RNA, proteins, and lipids. If this damage is not dealt with, metabolic imbalances occur and redox potentials are altered [1]. Abiotic stressors include a range of environmental influences, such as heat shock, ultraviolet light exposure, exposure to heavy metals and nutritional imbalances, starvation, and exposure to genotoxic substances. Biotic stress is caused by other organisms and viruses, and is dealt with by a range of cellular defense strategies and immune responses. Although attention has primarily focused on signaling pathways and protein networks, non-coding RNAs (ncRNAs) are increasingly implicated in the molecular mechanisms of these responses [2,3]. These ncRNAs can be roughly classified into two groups: small ncRNAs of 20–30 bases, such as microRNAs (miRNAs) [4]; and long ncRNAs of hundreds or thousands of bases, such as long intergenic ncRNAs (lincRNAs) [5]. Studies into long ncRNAs have revealed widespread roles for long ncRNAs in normal cellular processes, development, and physiology [6–8].

Many studies have described that miRNA expression patterns are altered in response to stress [9]. Although the long ncRNAs involved in cellular stress responses are just beginning to be investigated, there are few examples of long ncRNAs whose expression is altered by stress in humans. Alu RNA accumulates during heat shock, binding directly and tightly to RNA polymerase II (Pol II), and co-occupies the promoters of repressed genes along with Pol II, resulting in the repression of mRNA transcription [10]. The pericentromeric heterochromatin-associated satellite III sequence (Sat III) is activated upon heat shock and by chemical stressors, generating large amounts of polyadenylated ncRNAs that accumulate in nuclear stress bodies [11]. Heat shock RNA-1 (HSR1) ncRNA has been suggested to appear upon heat shock, and forms a complex with the translation elongation factor, eEF1A, stimulating the trimerization of the heat shock transcription factor, thereby promoting activation of heat shock response genes [12]. Serum starvation or treatment with translation inhibitors results in the arrest of cellular growth and increases in growth arrest-specific 5 (GAS5). GAS5 functions as a starvation- or growth arrest-linked riborepressor for the glucocorticoid receptor (GR) by binding to the DNA-binding domain of the GR [13,14]. The psoriasis susceptibility-related RNA gene induced by stress (PRINS) harbors two Alu elements, and is increased by many stressors such as ultraviolet-B irradiation, virus transfection, and translational inhibition [15]. LincRNA-p21 is induced by DNA damage, such as that caused by doxorubicin. This lincRNA acts to repress genes that are downregulated as part of the canonical p53 transcriptional response, and is necessary for p53-dependent apoptotic responses to DNA damage [16]. Numerous nuclear long ncRNAs, including MAGI2 antisense RNA 3 (MAGI2-AS3) and LOC730101, are increased by genotoxic

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Table 1
The 24 short-lived long ncRNAs that were investigate in this study.

Name	Accession No.	Length (nt)	$t_{1/2}$[a]
CDKN2B-AS1	NR_003529	3857	2.4
HOTAIR	NR_003716	2337	2.5
TUG1	NR_002323	7115	3.2
GAS5	NR_002578	651	2.6
MIR22HG	NR_028502	2699	2.4
FLJ43663	NR_015431	2964	2.4
LOC100216545	NR_024586	3615	2.3
LINC00667	NR_015389	3979	3.4
HCG18	NR_024052	6814	2.9
LOC550112	NR_015439	2301	3.2
LINC00662	NR_027301	2085	2.7
GABPB-AS1	NR_024490	4139	3.4
FLJ33630	NR_015360	2977	3.5
TTN-AS1	NR_038271	2033	2.6
LOC728431	NR_038842	997	3.0
LINC00473_v1	NR_026860	1832	2.4
LINC00473_v2	NR_026861	1123	2.4
FAM222A-AS1	NR_015431	1178	2.4
LINC00152	NR_024204	828	2.4
LINC0541471_v1	NR_015395	809	2.5
LINC0541471_v2	NR_024373	557	2.3
IDI2-AS1	NR_024628	1107	3.7
SNHG15	NR_003697	837	2.6
ZFP91-CNTF	NR_024091	3544	3.6

[a] These values are taken from a previous report [19].

agents [17]. These studies suggest that long ncRNAs are important and tightly controlled in response to stress, and have profound effects on transcription, RNA processing, and translation. However, alterations in RNA degradation when cells are subjected to stress remain to be elucidated.

Recently, two independent research groups reported that ncRNA half-lives vary over a wide range, and that they are comparable to those of mRNAs [18,19]. A genome-wide approach for determining RNA stability, known as 5′-bromo-uridine (BrU) immunoprecipitation chase-deep sequencing analysis (BRIC-seq) [19–21], revealed that ncRNAs with short half-lives (RNA half-life $t_{1/2} < 4$ h) included known regulatory ncRNAs. Those with long half-lives are a significant proportion of ncRNAs involved in housekeeping functions [19]. We hypothesized that the levels of long ncRNAs with short half-lives would be dramatically elevated owing to prolonged decay rates in response to stressors, and that there would be changes in RNA degradation pathways.

In this study, we sought to identify novel short-lived long ncRNAs that respond to chemical stressors in human cells. We identified six long ncRNAs that accumulate and prolong decay rate in response to model chemical stresses. These results suggest that distinct sets of nuclear long ncRNAs play roles in cellular defense mechanisms against specific stressors, and that particular long ncRNAs have the potential to be surrogate indicators for cellular stress response.

2. Materials and methods

2.1. Cell culture and stressor treatments

HeLa Tet-off (TO) cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37 °C/5% CO_2. HeLa TO cells (2×10^5 cells in a 12-well plate) were treated with cisplatin (final concentrations of 1, 10, or 100 μg/ml; Wako), cyclohexide (1, 10, or 100 μM; Biovi-

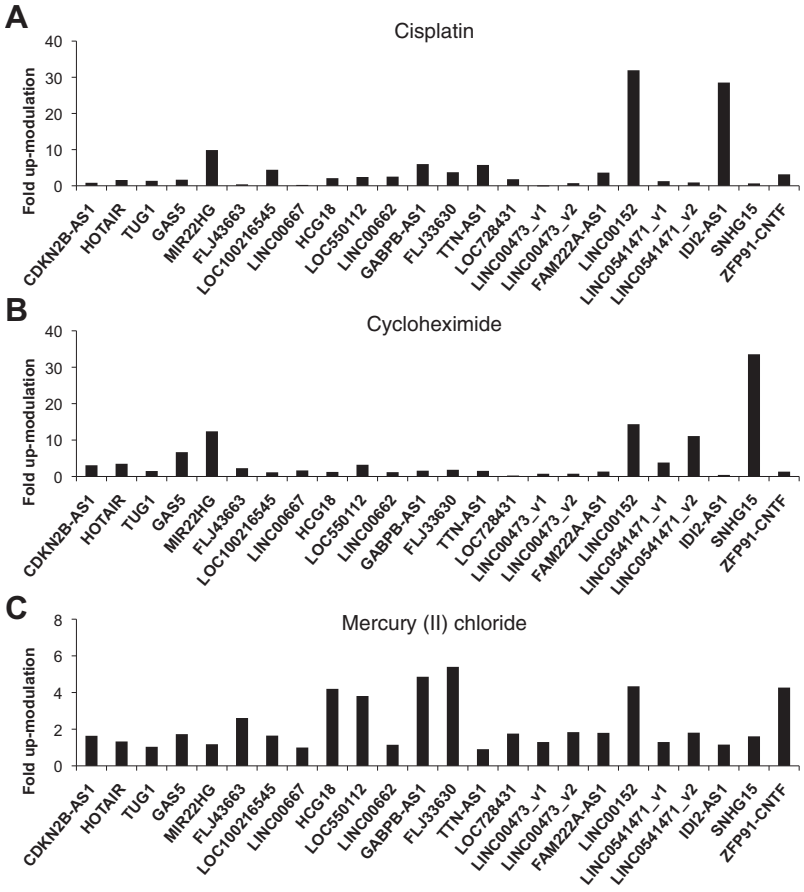

Fig. 1. Alterations in long ncRNA expression levels by three chemical stressors in HeLa Tet-off (TO) cells. HeLa TO cells were treated with (A) 100 μg/ml cisplatin, (B) 100 μM cycloheximide, or (C) 1 mg/ml mercury (II) chloride for 24 h. Expression levels of the indicated RNAs were determined by qPCR. GAPDH was used for normalization.

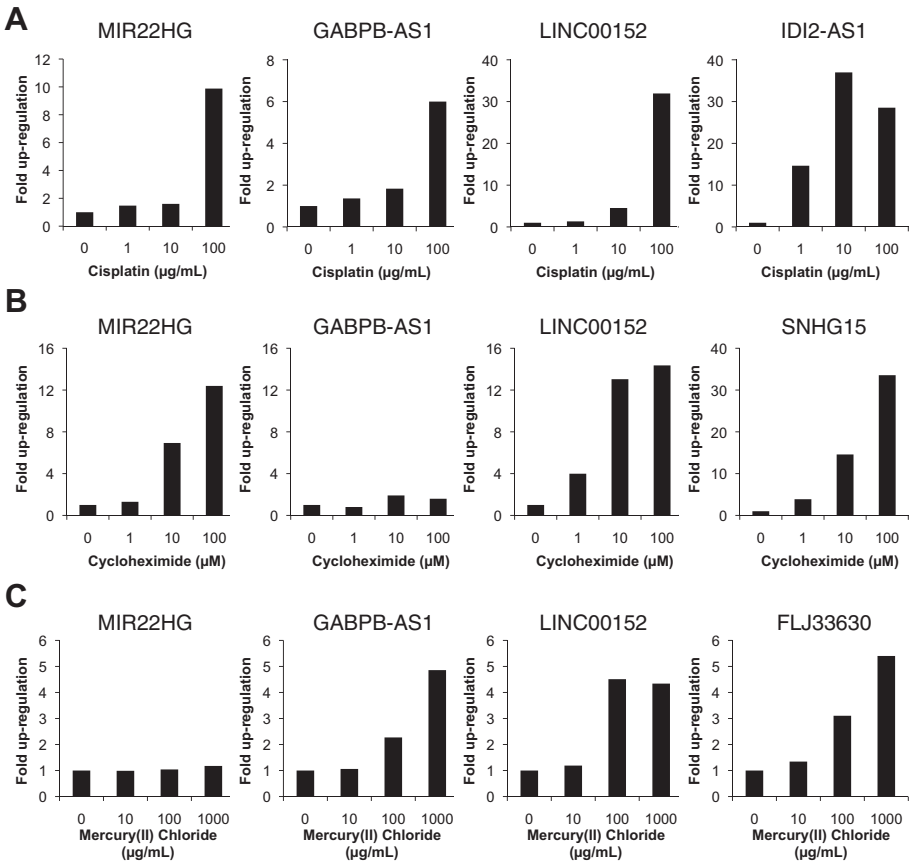

Fig. 2. Alterations in long ncRNA expression levels by three chemical stressors at various doses in HeLa TO cells. HeLa TO cells were treated with (A) cisplatin, (B) cycloheximide, or (C) mercury (II) chloride for 24 h. The expression levels of the indicated RNAs were determined by qPCR, with GAPDH used for normalization.

sion), or mercury (II) chrolide (10, 100, or 1000 μg/ml; Wako), and harvested 24 h after treatment.

2.2. Reverse-transcription quantitative real-time polymerase chain reaction (qPCR) assays

Total RNA was extracted from cells using RNAiso Plus (TaKaRa) according to the manufacturer's instructions. The isolated RNA was reverse transcribed into cDNA using the PrimeScript RT Master Mix (Perfect Real Time; TaKaRa). The resulting cDNA was amplified using the primer sets listed in Table S1, with GAPDH used for normalization. THUNDERBIRD SYBR qPCR mix (Toyobo) was used according to the manufacturer's instructions. Analysis of qPCR assays was conducted using a MyiQ2 (Bio-Rad).

2.2.1. 5-Ethnyluridine (EU) pulse labeling

Analysis of RNA half-lives were conducted by EU pulse labeling of RNA using the Click-iT Nascent RNA Capture Kit (Life Technologies) [14,22–24] according to the manufacturer's instructions, with some modifications. We added EU (300 μM) to culture medium and cells were incubated for an additional 24 h. At indicated time points after replacing EU-containing medium with EU-free medium, cells were harvested. Total RNA was isolated using RNAiso Plus (Takara). EU-labeled RNAs were biotinylated and captured using the Click-iT Nascent RNA Capture Kit (Life Technologies). To elute the EU-RNAs, magnetic beads were resuspended in 100 μl of buffer A (10 mM Tris–HCl, pH 7.4 and 6.25 mM EDTA). ISOGEN LS (300 μl; Nippon Gene) was added to the mixture, and EU-labeled RNAs were isolated according to the manufacturer's instructions; these isolated RNAs were used in qPCR assays.

3. Results

3.1. Screening of long ncRNAs in chemical stress response

We selected 24 short-lived long ncRNAs as outlined in a previous report [19]. These 24 ncRNAs were short lived ($t_{1/2}$ < 4 h), >200 nt, and fulfilled an established criterion for long ncRNA classification (Table 1). To investigate the response of the 24 long ncRNAs, we determined the alteration in their expression level following the treatment of HeLa TO cells with three stressors (cisplatin, cycloheximide, and mercury (II) oxide). Cisplatin inhibits DNA replication and transcription, cycloheximide inhibits translation, and mercury (II) chloride is a heavy metal (Fig. 1). Upon treatment with 100 μg/ml cisplatin, the expression levels of MIR22HG, GABPB-AS1, LINC00152, and IDI2-AS1 were increased (Fig. 1A). Upon treatment with 100 μM cycloheximide, the expression levels of MIR22HG, LINC00152, and SNHG15 were increased (Fig. 1B). Upon treatment with 1 mg/ml mercury (II) chloride, the expression levels of GABPB-AS1, FLJ33630, and LINC00152 were increased (Fig. 1C). Taken together, MIR22HG, GABPB-AS1, and LINC00152 responded to all three stressors; IDI2-AS1, SNHG15, and FLJ33630 specifically responded to cisplatin, cycloheximide, and mercury (II) chloride, respectively. These long ncRNAs have been identified in the human genome [25]; however, their functions remain unclear.

We also determined alterations in ncRNA expression levels following treatment with the three stressors at various doses (Fig. 2). As expected, MIR22HG, GABPB-AS1, LINC00152, and IDI2-AS1 levels increased with increasing concentrations of cisplatin (Fig. 2A). Expression levels of MIR22HG, LINC00152, and

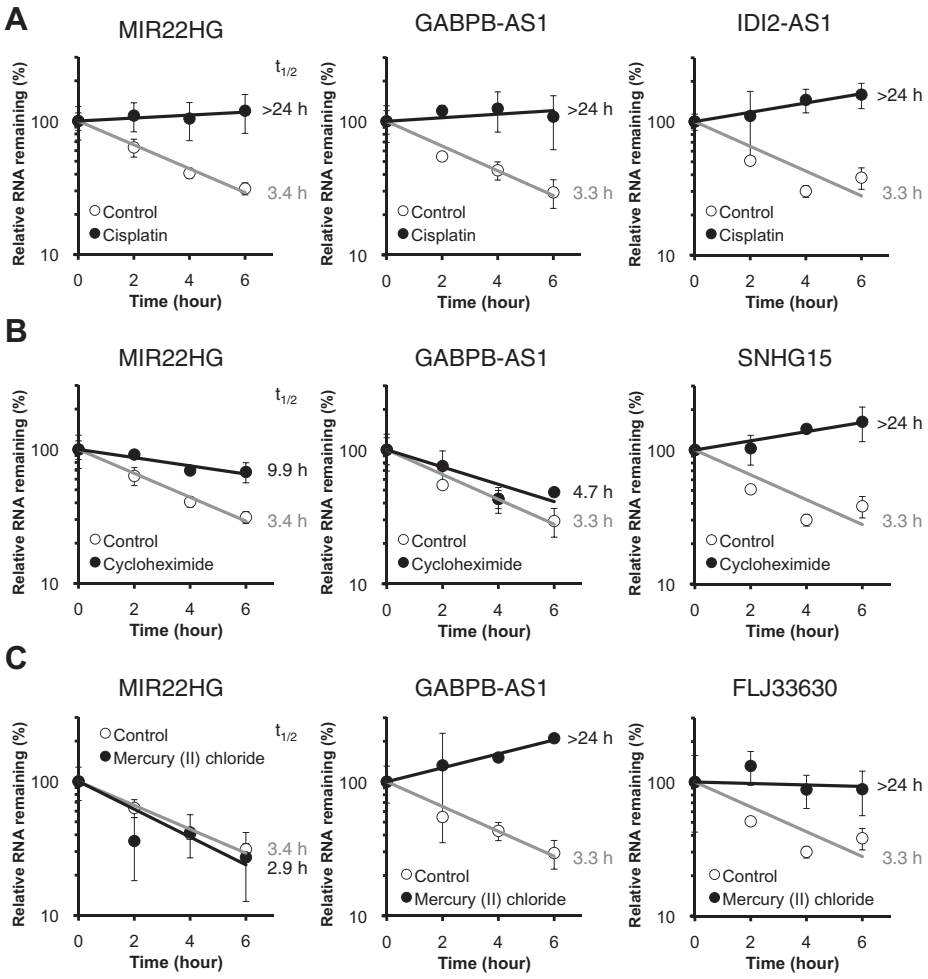

Fig. 3. Chemical stressors prolonged the decay rates of long ncRNAs in HeLa TO cells. HeLa TO cells were treated with (A) cisplatin, (B) cycloheximide, or (C) mercury (II) chloride. The decay rates of the indicated RNAs were determined in control (open circle and gray bar) and treated cells (solid circle and black bar). Relative quantitative values at 0 h were set to 100%. GAPDH was used for normalization. Values represent the mean ± SD obtained from two independent experiments.

SNHG15 increased in response to increasing concentrations of cycloheximide (Fig. 2B), while GABPB-AS1 remained unaltered. GABPB-AS1, LINC00152, and FLJ33630 levels were increased in reponse to increasing concentrations of mercury (II) chloride (Fig. 2C), while MIR22HG expression was unchanged. These data indicate that short-lived long ncRNAs respond to general or specific cell stresses.

3.2. Chemical stressors prolong the decay rates of long ncRNAs

To examine long ncRNA levels and their response to stressors when decay rates were prolonged, we determined the half-lives of the long ncRNAs in the presence or absence of stressors. Unfortunately, LINC00152 half-lives could not be determined because expression levels were too low in normal cells. Upon treatment with 100 μg/ml cisplatin, the $t_{1/2}$ of MIR22HG, GABPB-AS1, and IDI2-AS1 were increased from 3.4 to >24 h (Fig. 3A). Upon treatment with 100 μM cycloheximide, the $t_{1/2}$ of MIR22HG and SNHG15 were increased from 3.4 to >24 h, and GABPB-AS1 was unchanged (Fig. 3B). Upon treatment with 1 mg/ml mercury (II) chloride, the $t_{1/2}$ of GABPB-AS1 and FLJ33630 were prolonged from 3.4 to >24 h, and MIR22HG was not changed (Fig. 3C). These data suggest that the expression levels of short-lived long ncRNAs were elevated owing to prolonged decay rates in response to stressors, and that there were interruptions in RNA degradation pathways.

4. Discussion

In this study, we identified six short-lived long ncRNAs that respond to general or specific cell stressors. These long ncRNAs have the potential to be surrogate indicators of general or specific cell stresses. Recently, several long ncRNAs with distinct regulatory roles in responses to cellular stresses have been identified, but our present knowledge of the stress transcriptome is limited [2,3]. Long ncRNAs are associated with the specific nuclear speckled domain or nucleolus, and have effects on transcriptional and translational activities [26,27]. In addition, most environmental stresses affect multiple signaling pathways that sense environmental conditions and coordinate various cellular activities [28]. Depending on the nature of the damage and the cellular response, several of these signaling pathways likely lead to apoptosis of the affected cells. The role of long ncRNAs in these signaling pathways has remained largely unexplored. Although the functions of the identified long ncRNAs remain unknown, we demonstrated that RNA degradation is important for expression levels of the long ncRNAs during responses to cellular stress. We believe that this study will help to bridge the knowledge gap between digital genomic information and cellular function.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.006.

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