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ORIGINAL ARTICLE

Differential expression of long non-coding RNAs during genotoxic stress-induced apoptosis in HeLa and MCF-7 cells

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Abstract Long non-coding RNAs (lncRNAs) are emerging as new players in cancer as they are implicated in diverse biological processes and aberrantly expressed in a variety of human cancers. No data are available on their function under genotoxic stress-induced apoptosis. In this work, we assessed the behavior of some candidate lncRNAs (HOTAIR, MALAT1, TUG1, lincRNA-p21, GAS5, MEG3, PANDA, UCA1, ANRIL, and CCND1) during DNA damage-induced cell death in HeLa and caspase-3-deficient MCF-7 cells using bleomycin (BLM) and γ-radiation to induce DNA damage. Cells were incubated in the presence of BLM for 24 h or irradiated. Apoptosis was analyzed by measurement of oligonucleosomal fragmentation of nuclear DNA. Our results reveal that basal RNA expression levels as well as the changes in the lncRNA expression rates during genotoxic stress-induced apoptosis were cell-type and/or DNA-damaging agent-specific. Generally, we found that some of the RNA molecules (HOTAIR and MALAT1) are down-regulated while many of them (lincRNA-p21, GAS5, MEG3, ANRIL, and ncRNA-CCND1) are up-regulated and some others (TUG1, UCA1, and PANDA) not affected. The decline in the expression of HOTAIR (approx. twofold, $p < 0.01$) and MALAT1 (approx 1.6-fold, $p < 0.01$) was clearly evident in BLM-treated HeLa and MCF cells (only HOTAIR, fivefold, $p < 0.01$). For lincRNA-p21, ncRNA-CCND1, and MEG3, a similar up-regulation pattern was obvious in both cell lines where the increase was generally more pronounced in BLM-treated cells. Interestingly, the induction of ANRIL and GAS5 was mainly restricted to irradiated cells. In conclusion, our findings reveal a differential regulation of individual lncRNAs during genotoxic stress-induced apoptosis.

Keywords Long non-coding RNAs · Genotoxic stress · Apoptosis · Gene expression · HeLa cells · MCF-7 cells

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Introduction

Mammalian genomes produce a wide variety of non-coding RNA transcripts [1, 2]. In addition to classical "housekeeping" RNAs (e.g., ribosomal RNAs, transfer RNAs, and others) and well-defined microRNAs, long non-coding RNAs (lncRNAs) constitute a significant fraction of the untranslated RNA molecules. lncRNAs are transcribed by RNA polymerase II (RNA pol II), lack a significant open reading frame, are generally polyadenylated, and their length ranges from 200 nucleotides to $\sim$100 kilobases (kb). These mRNA-like molecules are pervasively transcribed and roughly classified as intergenic, intragenic/intronic, and antisense based on their position relative to the protein-coding genes [3]. They exhibit cis- or trans-regulatory capabilities and the mammalian genome encodes >1,000 lncRNAs that have been significantly conserved among mammals [2–4].

Gene expression patterns indicate that these lncRNAs are implicated in diverse biological processes, including nuclear architecture, regulation of gene expression, immune surveillance, or embryonic stem cell pluripotency. Recently, evidence revealing the molecular mechanisms by which these RNA species function has provided some insight into the functional roles they may play in

tumorigenesis [5]. Aberrant lncRNA expression participates in carcinogenesis by disrupting major biological processes, such as redirecting chromatin remodeling complexes or inactivating major tumor suppressor genes [5, 6]. Recent data suggest that transcription of lncRNAs can modulate gene activity in response to external oncogenic stimuli or DNA damage [7].

At present, no data are available on the role of lncRNAs in the genotoxic stress-induced intrinsic apoptotic pathway. In this study, we selected 10 candidate lncRNA molecules (lincRNAp21, TUG1, GAS5, HOTAIR, MALAT1, MEG3, PANDA, UCA1, ANRIL, and ncRNA-CCND1) and assessed their expression in relation to DNA damage-induced apoptosis. The reason for selecting these molecules was that many of them (lincRNA-p21; taurine up-regulated gene 1, TUG1; maternally expressed gene 3, MEG3; p21-associated ncRNA DNA damage activated, PANDA; and cyclin-D1-associated ncRNA, ncRNA-CCND1) have been reported to be associated with p53 and/or DNA damage [7–12]. Representatives of various biological functions such as normal growth arrest (the growth arrest-specific transcript 5, GAS5) [13], cell proliferation (urothelial cancer associated 1, UCA1) [11], epigenetic regulation (Hox transcript antisense intergenic RNA, HOTAIR and CDKN2B antisense RNA 1, ANRIL) [9, 14–16], or controlling alternative splicing (the metastasis-associated lung adenocarcinoma transcript 1, MALAT1) [17] were also included into the study. The impact of these molecules in genotoxic stress is not known.

Materials and methods

Study design

In the present study, we aimed to determine the association of the long non-coding RNA molecules with genotoxic stress-induced, intrinsic apoptotic cell death. For this purpose, we used two different cell lines as model cell systems: the HeLa human cervix adenocarcinoma cell line and the mammary adenocarcinoma cell line MCF-7. The latter cell line is caspase-3 deficient and resistant to radiation-induced intrinsic cell death [18]. Because of the similar chemistry of the DNA strand breaks [19], and similar qualitative and quantitative chromosomal aberrations [20], we used bleomycin (BLM) and γ-radiation to induce genotoxic stress. We hypothesized that RNA molecules in the two cell lines would exhibit different responses during DNA damage-induced apoptosis when exposed to damaging agents. In line with the caspase-3 status in MCF-7 cells and the fact that caspase-3 is required for oligosomal DNA fragmentation [21], we analyzed the release of fragmented nuclear DNA into cytoplasm as the index of apoptotic cell death.

Cell culture

HeLa and MCF-7 cells were purchased from the German Resource Centre for Biological Materials (DSMZ) (DSMZ # ACC 57 and 115, respectively). Cells were grown in the DMEM culture medium containing $NaHCO_3$ (3.7 g/L), glucose (1 g/L), and stabile glutamine (Biochrom, Berlin, Germany) supplemented with 10 % FCS (Biochrom) and antibiotics under standard conditions (37 °C and 5 % CO_2 humidity). Cells were sub-cultivated at a confluency of approx. 80 %. For experiments, cells with a passage number <20 were used.

DNA damage induction

Twenty-four hours after plating, 1×10^5 cells were incubated in 60 mm petri dishes in the presence or absence of BLM (Applichem, Germany) for 24 h or alternatively the cells were irradiated to total doses of 0, 2, and 5 Gy using a Cobalt-60 γ-ray source at a dose rate of 200 cGy/min and kept under standard growth conditions for further 24 h.

Detection of apoptotic cell death

To detect and quantify apoptotic cell death, we used the Cell-Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany). This photometric enzyme immunoassay is used for the specific in vitro detection and quantitation of mono- and oligonucleosomes that are released into the cytoplasm from apoptotic cells at early stages of cell death [22]. The assay was performed according to the instructions of the manufacturer using cytoplasmic lysates of treated and untreated cells in duplicate, and relative concentrations of cytoplasmic nucleosomes were determined from the mean absorbance values.

p53 ELISA

We determined the accumulation of the p53 protein upon exposure of cells to genotoxic agents by a pan-p53 ELISA assay (Roche). The assay was performed according to the manufacturer's instructions using cytoplasmic lysates, and relative p53 concentrations were determined from the mean absorbance values using a calibration plot.

Gene expression analysis

Total RNA was extracted from cells using the High Pure RNA Isolation Kit (Roche) and converted to cDNA using the Revert Aid FirstStrand cDNA Synthesis kit (Fermantas, Vilnius, Lithuania). Five microliters (approx. 500 ng) of cDNA were used in quantitative PCR (qPCR),

and the results were standardized to the housekeeping gene *GAPDH*. Primers were used according to Huarte et al. [8], Khalil et al. [9], Mourtada-Maarabouni et al. [23], Wang et al. 2008 [11], Yap KL et al. 2008 [16], Zhou et al. [10], Hung et al. 2011 [7] and purchased from Integrated DNA Technologies (Coralville, IA, USA). Primers for the amplification of *ATM*, *p21,* and *GADD45* were designed using the Universal Probe Library Software (Roche). PCR efficiencies of primers were determined by the amplification of serially diluted template DNA where we obtained linear curves (correlation coefficient, $r = 0.98$) with decreasing concentrations.

qPCR was performed in the LightCycler 480 Instrument (Roche) using SYBR Green I (Roche) as the fluorescence molecule. Samples with a Ct > 40 were considered negative. Amplification of the appropriate product was confirmed by melting curve analysis following amplification.

Results of three independent cell culture experiments were evaluated to calculate the average value of each given parameter. Basal levels of p53, apoptosis, or gene expression were determined using the BLM-treated cells as the time outside the incubator was shorter in this option when compared to irradiated cells. Statistical analysis was performed using Student's *t*-test, and $p < 0.05$ was considered statistically significant.

Results

In preliminary experiments, we determined the doses of BLM to induce significant levels of cytoplasmic release of DNA fragments for an incubation time of 24 h. Doses of 0, 35, and 70 μg/mL were appropriate to achieve this goal and were used in subsequent experiments, while for γ-ray irradiation, intermediate (2 Gy) and high doses (5 Gy) were used. We first confirmed a DNA damage response by investigating some DNA damage response genes. For this purpose, p53 was measured at the protein level, while the ataxia telangiectasia-mutated (*ATM*) kinase, *p21,* and the growth arrest and DNA damage-inducible genes (*GADD45*) were investigated at the mRNA level. Basal p53 level was about sixfold higher in the MCF-7 cells than in HeLa cells. A similar accumulation pattern was observed for both cell lines and both DNA-damaging agents (Figs. 1, 2). Similar to p53, mRNA levels of *p21* and *GADD45* accumulated upon damage induction in both cell lines (Figs. 1, 2), while for *ATM,* no clear change was observed. In contrast to the accumulation of DNA response proteins, the apoptotic cell death rate was different for the two DNA-damaging agents. As seen in Fig. 3a and b, BLM induced highly significant levels of fragmented DNA release into the cytoplasm in both cell lines ($p < 0.01$), while the extent of γ-radiation-induced cell death was substantially lower and was restricted to HeLa cells (approx. threefold at 5 Gy, $p = 0.014$). In MCF-7 cells, no change was observed when compared to untreated cells.

Next, we determined the expression of lncRNAs during DNA damage-induced apoptosis. There were remarkable differences between the basal levels of RNA molecules. MALAT1 that is broadly expressed in human tissues [17] displayed substantially high expression levels in both cell lines, while the majority of investigated lncRNAs were expressed at very low levels. Figure 3c and d show the relative basal expression levels that were higher than 1 only

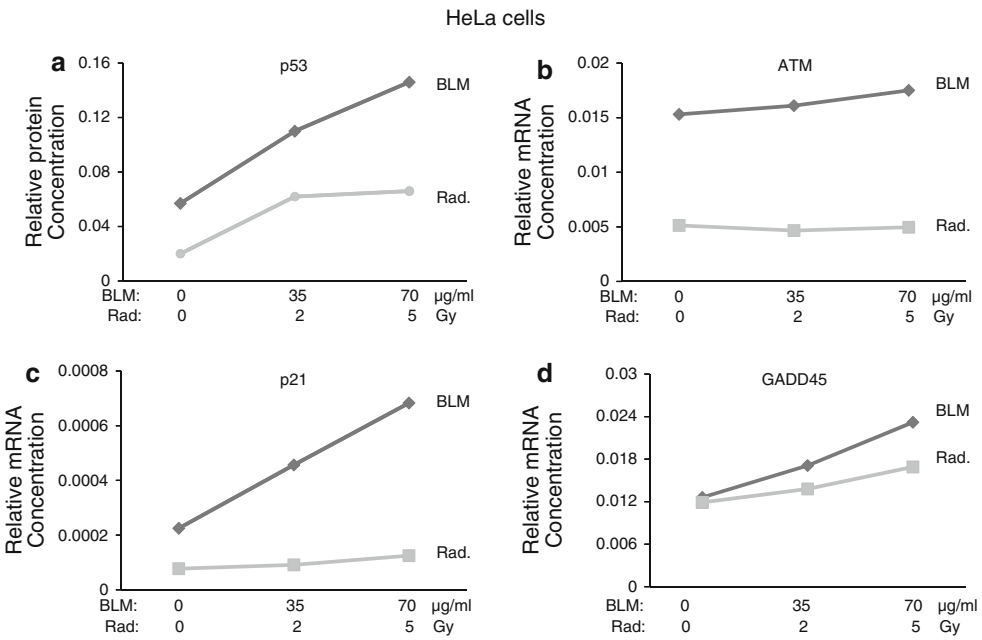

Fig. 1 Expression of DNA damage response genes in HeLa cells during genotoxic stress-induced apoptosis. **a** p53 was measured at protein levels from cytoplasmic lysates of BLM-treated and irradiated cells used to quantitatively determine the accumulation of p53 protein. **b–d** The expression of *ATM*, *p21,* and *GADD45* were investigated at mRNA level through qPCR. Mean levels of three independent experiments are given

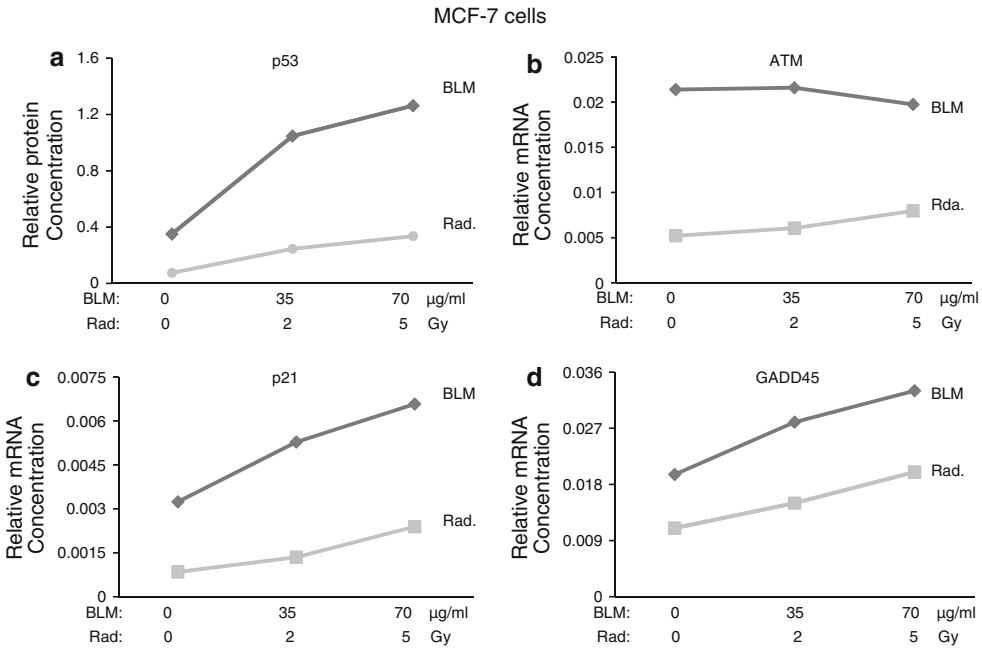

Fig. 2 Expression of DNA damage response genes in MCF-7 cells during genotoxic stress-induced apoptosis. **a** p53 at protein level, **b–d** Expression of *ATM*, *p21,* and *GADD45* at mRNA level. Mean levels of three independent experiments are given

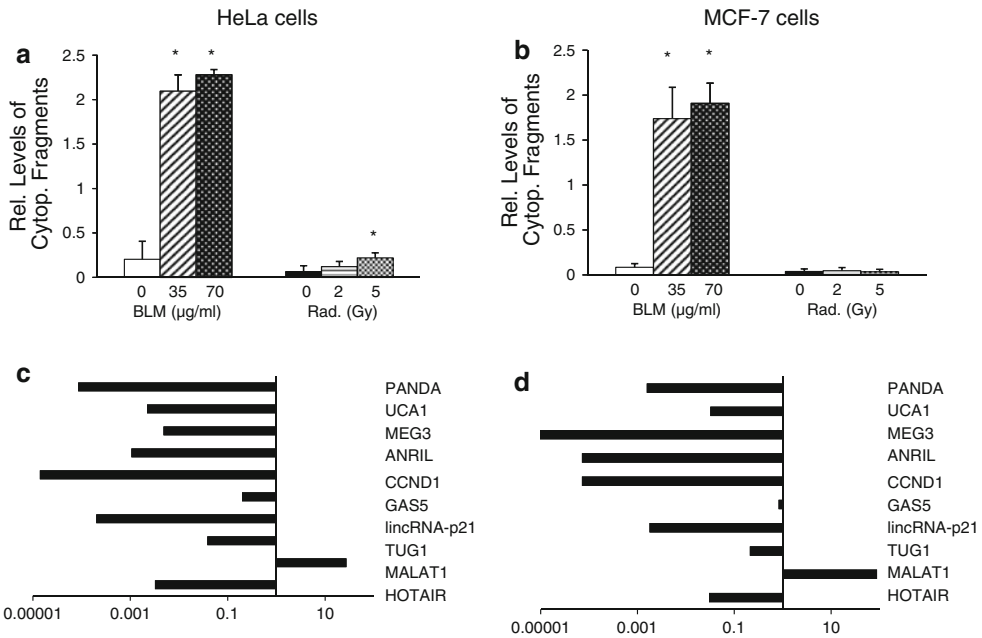

Fig. 3 Apoptosis levels and basal expression levels of lncRNAs. Cytoplasmic release of nuclear DNA fragments was measured by ELISA in Hela (**a**) and MCF-7 (**b**) cells during genotoxic stress. Results of three independent cell culture experiments were evaluated to calculate the average value of relative gene expression. Each column represents mean + S.E and *indicates statistically significant differences compared to untreated cells. BLM, bleomycin; Rad, radiation. **c**, **d** Basal expression levels of lncRNAs in HeLa and MCF-7 cells. For comparison of basal levels, we considered BLM-treated cells. Please note the axis representing relative expression is logarithmic

for MALAT1, while it was extremely low for the majority of the other molecules.

Interestingly, the response of lncRNAs to genotoxic stress was cell-type and/or DNA-damaging agent-specific. We found two long RNA molecules, HOTAIR and MALAT1, to be clearly down-regulated in a dose-dependent way. The decline in HOTAIR expression was restricted to BLM treatment and was more pronounced in MCF-7 cells (approx. fivefold, $p < 0.01$) than in HeLa cells (approx twofold for the highest dose, $p < 0.01$) (Figs. 4a and 5a). For MALAT1, we observed a statistically significant dose-dependent decrease (approx 1.6-fold for the highest dose, $p < 0.01$) in BLM-treated HeLa cells but not in BLM-treated MCF-7 cells and irradiated cells (Figs. 4b, 5b). Five of the 10 molecules investigated (lincRNA-p21, GAS5, ncRNA-CCND1, ANRIL, MEG3) were up-regulated to various levels, at least in one cell-type or in response to one DNA-damaging agent (Figs. 4, 5), while for TUG1, PANDA, and UCA1, no significant changes were detected. For lincRNA-p21 (Figs. 4e, 5e), ncRNA-CCND11 (Figs. 4f, 5f), and MEG3 (Figs. 4h, 5h), a similar up-regulation pattern was obvious in both cell lines where

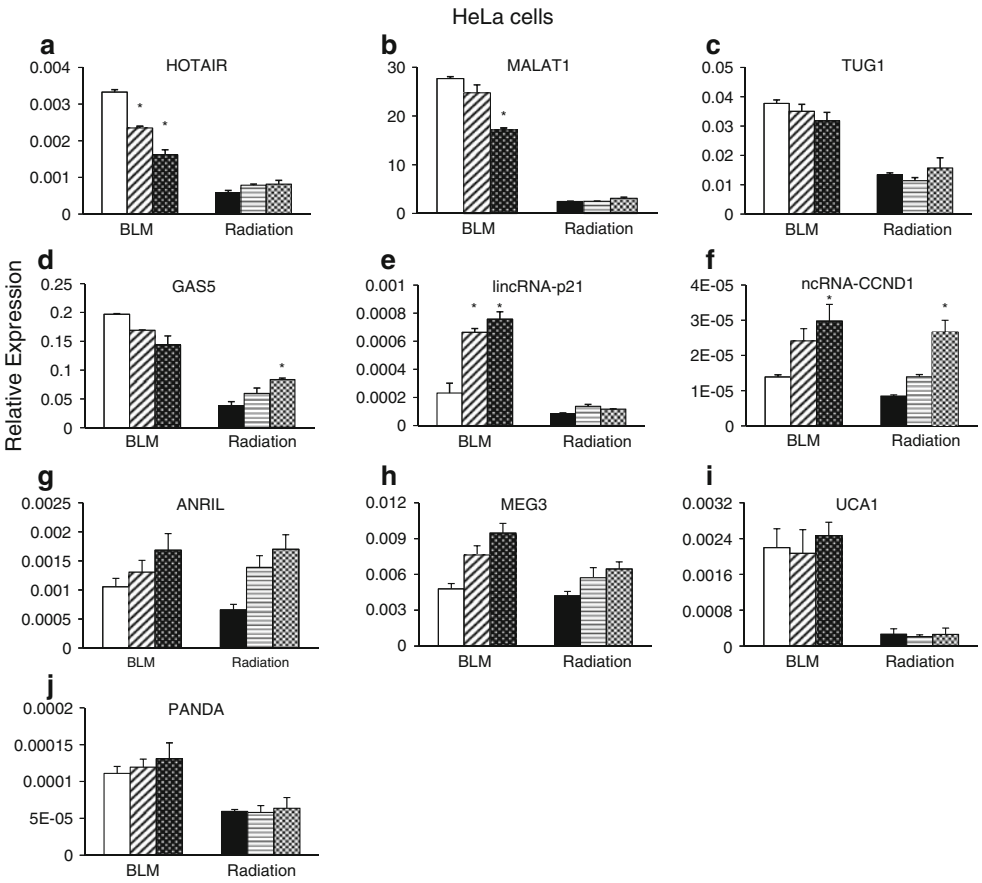

Fig. 4 Expression of lncRNAs during genotoxic stress-induced apoptosis in HeLa cells. Given are HOTAIR (**a**), MALAT1 (**b**), TUG1 (**c**), GAS5 (**d**), lincRNA-p21 (**e**), ncRNA-CCND1 (**f**), ANRIL (**g**), MEG3 (**h**), UCA1 (**i**), and PANDA (**j**). Results of three independent cell culture experiments were used to calculate the average value of relative gene expression. Each column represents mean + SE and * indicates statistically significant differences compared to untreated cells. Doses of bleomycin and radiation were as above

the increase was generally more pronounced in BLM-treated cells. Up-regulation of ANRIL was mainly restricted to irradiated cells (Figs. 4g, 5g) where the increase was more than tenfold ($p < 0.01$) at 5 Gy in MCF-7 cells. A similar exception was also GAS5 (Fig. 4d); its expression was induced in irradiated HeLa (twofold at 5 Gy, $p = 0.018$) but not in irradiated MCF-7 cells or BLM-treated cells.

Discussion

Although lncRNAs may have an impact on various human diseases including cancer, the basis of their molecular mechanism is still beginning to be elucidated [15]. Likewise, no data are available on their role during genotoxic stress-induced apoptosis. In this work, we analyzed the behavior of lncRNA molecules during DNA damage-induced cell death in HeLa and caspase-3-deficient MCF-7 cells. By looking at some response genes such as *p53*, *p21*, and *GADD45,* we confirmed the presence of DNA damage response for both damaging agents in both cell lines. BLM treatment caused significant levels of DNA fragmentation in both cell lines while the extent of the cell death induced by radiation was much lower and was confined to HeLa cells. This is in line with the well-known resistance to radiation-induced intrinsic cell death and the caspase-3 status of MCF-7 cells [18, 24]. The differences in the apoptotic responses to BLM and radiation in the HeLa cells may be attributed to the continuous exposure of the cells to BLM for 24 h, while the irradiated cells had chance to counteract the stress by cell cycle arrest and DNA damage repair. On the other hand, it is possible that BLM-induced cell death in caspase-3-deficient MCF-7 cells leads to DNA fragmentation via a caspase-3-independent pathway, possibly via caspase-7. Consistent with our finding, oligonucleosomal fragmentation of nuclear DNA in the MCF-7 cells by various stimuli has been reported in previous studies [25, 26].

Recent experimental data suggest that lncRNA expression levels appear to be generally lower than protein-coding genes [5]. For example, only less than two copies of ncRNA-CCND1 per cell were detected in various human cell lines [27]. Consistent with this, we detected very low amounts of RNA molecules in HeLa and MCF cells, except for MALAT1. Like their levels, also the response of lncRNAs to genotoxic stress was distinct and cell-type and/or DNA-damaging agent-specific. During stress conditions, we observed that some lncRNAs (HOTAIR, MALAT1) are down-regulated, whereas some others (lincRNA-p21, ncRNA-CCND1, MEG3, ANRIL) were up-regulated or not

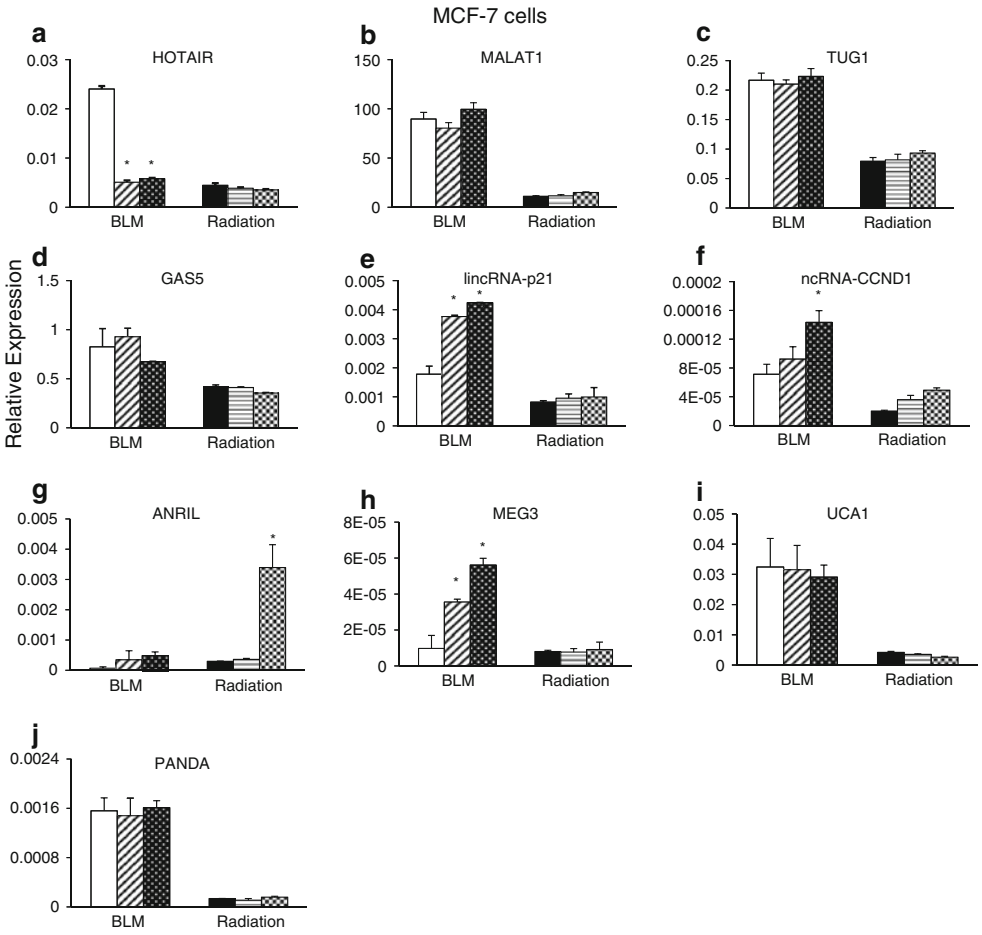

Fig. 5 Expression of lncRNAs during genotoxic stress-induced apoptosis in MCF-7 cells. Expression of lncRNAs during genotoxic stress-induced apoptosis in HeLa cells. Given are HOTAIR (**a**), MALAT1 (**b**), TUG1 (**c**), GAS5 (**d**), lincRNA-p21 (**e**), ncRNA-CCND1 (**f**), ANRIL (**g**), MEG3 (**h**), UCA1 (**i**), and PANDA (**j**). Results of three independent cell culture experiments were used to calculate the average value of relative gene expression. Each column represents mean + SE and *indicates statistically significant differences compared to untreated cells

affected (TUG1, PANDA, UCA1). Down-regulation was straightforward for HOTAIR and MALAT1 and restricted to BLM-treated cells. The difference between BLM-treated cells and irradiated cells is likely to be related to the extent of DNA damage as demonstrated by different apoptosis levels between these two conditions. Considering the role of HOTAIR as a modulator of chromatin by binding the polycomb repressive complex to methylate histones at multiple Hox loci and its aberrant expression in cancer to promote invasion and metastasis [9, 14, 15, 28], and MALAT1 acting as a regulator of alternative splicing [29], the down-regulation of these molecules under stress conditions may contribute to maintain the stability of chromatin and provide advantage in favor of apoptosis to avoid genomic instability. In contrast to HOTAIR and MALAT1, many of the investigated molecules (lincRNA-p21, GAS5, ncRNA-CCND1, ANRIL, MEG3) were induced to various levels during genotoxic stress-induced apoptosis. Up-regulation of these molecules may help the cells respond to genotoxic stress and possibly induce genes required for apoptotic cell death. Induction of these molecules was, however, not unique to all treatment conditions or to both cell lines. For instance, lincRNA-p21 that is induced by p53 [8] was not up-regulated in irradiated cells though p53 accumulated. Thus, variations in the expression patterns of long RNA molecules may be related to their biological function, the extent of DNA damage or to the cell-type or to other factors not defined yet. ncRNA-CCND1 is an example of promoter-related lncRNA, which is transcribed from the promoter of the cyclin D1 gene (*CCND1*) [11] and represses its transcription by modifying RNA binding protein in *cis*. Enhanced expression of ncRNA-CCND1 may repress the transcription of *CCND1* upon DNA damage [12] blocking proliferation of damaged cells. Also, PANDA is a promoter-related RNA expressed from the *CDKN1A* promoter and is induced in a p53-dependent manner [7]. It interacts with the transcription factor NF-YA to limit expression of pro-apoptotic genes. However, in our series, we detected no remarkable change in PANDA expression despite p53 accumulation. It is plausible that PANDA induction by p53 may be dependent on cellular context whether or not apoptosis comes into play for damaged cells. Recent data suggest that MEG3 could act as a tumor suppressor whose action may be mediated by p53 [10]. Expression of MEG3 in tumor cells results in growth suppression, p53 protein increase, and activation of

downstream p53 targets [30]. In concordance with this picture, we found a clear induction of MEG3 by genotoxic stress. In contrast to previous up-regulated RNA molecules, induction of ANRIL was mainly restricted to irradiated cells. ANRIL is transcribed from the INK4a locus and is involved in epigenetic regulation of INK4b/ARF/INK4a tumor suppressors [16]. It is assumed that ANRIL binds to and recruits PRC2 to repress the expression of genes from this locus [31]. In damaged cells, suppression of this locus may be required for growth arrest. A similar behavior was observed for GAS5 that is induced in only irradiated HeLa cells. As GAS5 is a specific inducer of cell cycle arrest [13], interruption of the cell cycle in irradiated HeLa cells may result in lower cell death.

In conclusion, this is the first study to describe a significant response of lncRNAs to stress induction where especially HOTAIR and MALAT1 are down-regulated and molecules such as lincRNA-p21, ncRNA-CCND1, ANRIL, and MEG3 are up-regulated. In future work, high-throughput techniques should be applied to obtain a global view on the changes of lncRNA molecules and to elucidate the mechanisms of regulation of individual lncRNAs.

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Conflict of interest None.

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