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Time, Dose and Ataxia Telangiectasia Mutated (ATM) Status Dependency of Coding and Noncoding RNA Expression after Ionizing Radiation Exposure

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Studies of gene expression have proved important in defining the molecular mechanisms of radiation action and identifying biomarkers of ionizing radiation exposure and susceptibility. The full transcriptional response to radiation is very complex since it also involves epigenetic mechanisms triggered by radiation exposure such as modifications of expression of noncoding RNA such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) that have not been fully characterized. To improve our understanding of the transcriptional response to radiation, we simultaneously monitored the expression of ten protein-coding genes, as well as 19 miRNAs and 3 lncRNAs in a time- and dose-dependent manner in stimulated human T lymphocytes obtained from two healthy donors (C1 and C2) and one patient with ataxia telangiectasia (AT), which is a well characterized radiosensitivity disorder. After 2 Gy X irradiation, expression levels were monitored at time points ranging from 15 min up to 24 h postirradiation. The majority of genes investigated responded rapidly to radiation exposure, with the peak up-regulation (*CDKN1A, SESN1, ATF3, MDM2, PUMA* and *GADD45A*) or down-regulation (*CCNB1*) occurring 2–3 h postirradiation, while *DDB2, FDXR* and *CCNG1* responded with slower kinetics reaching a peak of expression between 5 and 24 h. A significant modification of expression after radiation exposure was observed for *miR-34a-5p* and *miR-182-5p*, with an up-regulation occurring at late time points reaching two to threefold at 24 h. Differences between two donors in *miR-182-5p* response to radiation were detected: for C2, up-regulation reached a plateau-phase around 5 Gy, while for C1, up-regulation was at its maximum around 3 Gy and then decreased at higher doses. Among the three lncRNAs studied, *TP53TG1* demonstrated a weak up-regulation, reaching a maximum of 1.5-fold at 24 h after radiation exposure. Conversely, *FAS-AS1* was up-regulated up to fivefold by 5 Gy irradiation. Our results indicate that expression of the majority of protein-coding genes allows discrimination of the AT from healthy donors when analyzed at 2 h. However, differences in expression between AT and healthy donors are no longer detectable 24 h postirradiation although, interestingly, linear dose responses for some of the genes studied are obtained at this time point. Furthermore, our study shows that miRNAs *miR-34a-5p* and *miR-182-5p* are responsive to radiation exposure in a dose- and time-dependent manner. Additionally, to the best of our knowledge, this is the first study to report that *FAS-AS1* lncRNA is up-regulated by radiation exposure in an ATM-dependent fashion in human T lymphocytes.

INTRODUCTION

Biological research to assess the environmental health risks associated with ionizing radiation can help characterize and broaden our understanding of the actions of radiation on biological processes such as transcription. All living cells execute their functions through the fundamental mechanism of transcription of their genome. There is a growing body of evidence to suggest that while the majority of the mammalian genome is actively transcribed, only about 2% of the transcriptome encodes for proteins (*1–3*). The ''dark matter'' of the genome consists of noncoding RNAs, of which there are several groups: well known tRNAs and rRNAs; small nucleolar and nuclear RNAs implicated in various steps of RNA processing; miRNAs, which are post-transcriptional regulators of gene expression; piRNAs involved in epigenetic silencing of transposons in the germ line; and a large group of long noncoding RNAs (lncRNAs) the functions of which are just starting to be discovered.

The first mammalian radiation-induced protein-coding gene, i.e., tumor necrosis factor (*TNF*), was reported in the late 1980s (*4*). With the development of microarray technology that enables screening of hundreds of genes simultaneously (*5*), it became clear that many more genes are modulated in response to radiation exposure (*6–13*), mostly in a TP53-dependent manner. Gene expression changes after

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exposure to radiation are now well documented in human blood (*14*), even after low-dose exposures (*15*).

MicroRNAs (miRNAs) are a class of small noncoding RNAs that post-transcriptionally regulate gene expression (*16*). Since their discovery, miRNAs have been implicated in virtually every process investigated in the cell. miRNAs appear to be essential for cellular responses to radiation exposure, as global miRNA reduction achieved by down-regulation of DICER reduces cell survival after radiation exposure mediated by impaired cell cycle checkpoint activation and increased apoptosis (*17*). In 2007, He *et al*. reported that miRNAs belonging to the *miR-34* family were induced in a TP53-dependent manner by radiation in a variety of mouse tissues (*18*). This published finding inspired the search for other radiation-responsive miRNAs (*19–22*). The radiation-induced miRNA response depends on radiation dose, time post exposure, genetic background (*23–26*), the tissue being investigated and gender (*27–29*).

The definition of long noncoding RNA is very broad and unspecific: every RNA molecule longer than 200 nucleotides which is not ribosomal RNA or transfer RNA and lacks significant protein-coding potential is defined as a lncRNA (*30*). Although the functions of the overwhelming majority of lncRNAs are still unknown, a small characterized fraction seems to play very diverse roles in genomic imprinting (*31*), chromosome X dosage compensation (*32*), growth arrest (*33*), control of pluripotency and differentiation (*34*), apoptosis (*35*), gene expression (*36*) and DNA methylation (*37*), to name just a few.

The lncRNA concept is relatively new in radiation biology and only a few radiation-responsive lncRNAs have been identified so far. The majority of experiments were performed using radiomimetic drugs, which induce double-strand breaks (DSBs) such as doxorubicin, bleomycin or etoposide. The first lncRNA showing modification of expression upon induction of DSBs was TP53 target 1 (nonprotein coding) (*TP53TG1*) (*38*). Several other lncRNAs have been found to be up-regulated after doxorubicin treatment in various cell lines, such as: tumor protein p53 pathway corepressor 1 (*Trp53cor1*) (*39*); nonprotein-coding RNA, associated with MAP kinase pathway and growth arrest (*NAMA*) (*40*); promoter of CDKN1A antisense DNA damage activated RNA (*PANDAR*) (*41*); long intergenic nonprotein-coding RNA, which regulates reprogramming (*linkRNA-RoR*) induced in a TP53-dependent manner after DNA damage (*42*); urothelial cancer associated 1 (nonprotein coding) lncRNA (*UCA1*) up-regulated in a TP53-independent manner in human breast cancer cell line (*43*); and E2F1-regulated lncRNA XLOC 006942 (*ERIC*) (*44*). Wan *et al*. reported significant ATM-dependent up-regulations of CDKN2B antisense RNA 1 (*CDKN2B-AS1*, also known as *ANRIL*) (*45*) and JADE1 adjacent regulatory RNA (*JADRR*) (*46*) after treatment with radiomimetic drugs. Other novel lncRNAs whose expression is modified after doxorubicin treatment have unknown functions (*47*).

The first reported lncRNA induced by radiation exposure was *lncRNA-CCND1*, which forms a ribonucleoprotein complex and represses *CCND1* transcription after DNA damage (*48*). Chaudhry *et al*. showed that SOX2 overlapping transcript (nonprotein coding) (*SOX2-OT*) expression is modified more than twofold by radiation exposure (*49*). Özgür *et al*. observed cell line-dependent differences in expression of lncRNAs playing roles in TP53 pathway or DNA damage after gamma-radiation exposure or bleomycin treatment in human cervical and breast cancer cell lines (*50*). Interestingly, contrary to a previous report (*41*), *PANDAR* was not responsive to bleomycin or radiation treatment in either of the cell lines, possibly indicating tissue-specific transcriptional response to DNA damaging agents (*50*).

To characterize the responses of noncoding RNAs to radiation, the detailed temporal- and dose-response characteristics of candidate transcripts must be understood. As we have recently shown, for some genes there is significant variability in the transcriptional response to radiation within the healthy population (*15*). There are also individuals in certain populations, such as ataxia-telangiectasia (AT) patients, who display a characteristic phenotype, including hypersensitivity to ionizing radiation and chromosomal instability (*51*). AT patients have an autosomal, recessive disorder, and while these cases are very rare, the estimated frequency of heterozygous carriers of the responsible gene, ATM, who may have increased cancer risk due to increased radiation sensitivity as demonstrated by cellular experiments, is around 0.5% in the UK (*52*).

In this study, we investigated time- and dose-dependent changes in the expression of several radiation-responsive protein-coding genes, lncRNAs and miRNAs, in cultured human T lymphocytes derived from two healthy donors and one AT patient. Our findings showed that *FAS-AS1* lncRNA is up-regulated by radiation exposure in human T lymphocytes, which to the best of our knowledge, has not been previously reported.

MATERIALS AND METHODS

Samples

Blood was collected from two healthy female donors (age range 37–43 years old). Blood lymphocytes were separated on Histopaque®-1077 (Sigma-Aldrich, Poole, UK) and were used to produce short-term T-cell cultures (named C1 and C2). AT T lymphocytes obtained from one individual were kindly provided by Dr. C. Arlett, University of Sussex (Brighton, UK) (*53*).

Cell Growth

T-lymphocyte cultures were prepared as follows. Briefly, after thawing, normal human T lymphocytes were seeded at 3×10^5 cells/ml in stimulating growth medium (SR10) comprised of RPMI 1640 (Dutch modification) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μ*M* 2-mercaptoethanol (Invitrogen Ltd., Paisley, UK), 250 IU/ml recombinant interleukin-2 (Novartis Pharmaceuticals UK Ltd., Camberley, UK) and 0.4 μg/ml phytohaemagglutinin (PHA), (Remel™ Products, Thermo Fisher

TABLE 1
Primers and Probes

Gene	Accession no.	Primers	Probe
ATF3	NM_001030287 NM_001040619 NM_001674	**F** - AGGTTTGCCATCCAGAACAA **R** - CTGACAGTGACTGATTCC	CCTCTGCCACCGGATGTCCTCT
BBC3 (*PUMA*)	NM_014417.3 NM_001127240.1 NM_001127241.1 NM_001127242.1	**F** - CGGAGACAAGAGGAGCAG **R** - GGAGTCCCATGATGAGATTG	CCCTCACCCTGGAGGGTCCTGT
CCNB1	NM_031966.2	**F** - ATAAGGCGAAGATCAACATGGC **R** - TTTGTTACCAATGTCCCCAAGAG	CGCAAAGCGCGTTCCTACGGCC
CCNG1	NM_004060.3 NM_199246.1	**F** - GGAGCTGCAGTCTCTGTCAAG **R** - TGACATCTAGACTCCTGTTCCAA	AACTGCTACACCAGCTGAATGCCC
CDKN1A	NM_000389.3, NM_078467.1	**F** - GCAGACCAGCATGACAG **R** - TAGGGCTTCCTCTTGGA	TTTCTACCACTCCAAACGCCGGCT
DDB2	NM_000107	**F** - GTCACTTCCAGCACCTCACA **R** - ACGTCGATCGTCCTCAATTC	AGCCTGGCATCCTCGCTACAACC
FAS-AS1	NR_028371.1	**F** - CCTCATTTCGCCATCTGTA **R** - GCATAGCGAGAGAAGTGTT	ACTACATGGCTCTCGTGAGAATCC
FDXR	NM_024417 NM_004110	**F** - GTACAACGGGCTTCCTGAGA **R** - CTCAGGTGGGGTCAGTAGGA	CGGGCCACGTCCAGAGCCA
GADD45A	NM_001924.2	**F** - CTGCGAGAACGACATCAAC **R** - AGCGTCGGTCTCCAAGAG	ATCCTGCGCGTCAGCAACCCG
HPRT1	NM_000194.2	**F** - TCAGGCAGTATAATCCAAAGATGGT **R** - AGTCTGGCTTATATCCAACACTTCG	CGCAAGCTTGCTGGTGAAAAGGACCC
MDM2	NM_002392	**F** - CCATGATCTACAGGAACTTGGTAGTA **R** - ACACCTGTTCTCACTCACAGATG	CAATCAGCAGGAATCATCGGACTCAG
PANDAR	http://www.lncrnadb.org NR_109836.1	**F** - GTCCTGATGCAGACCATAAA **R** - GATAGCTGGAAAGCTGAGAG	CCTTCAGAGGTGGTCCAGATATGT
SESN1	NM_014454	**F** - GCTGTCTTGTGCATTACTTGTG **R** - CTGCGCAGCAGTCTACAG	ACATGTCCCACAACTTTGGTGCTGG
TP53TG1	NR_015381.1	**F** - CCAAATGAGCTGTCCTAACT **R** - AGAGTGCCTTCTAGATCCT	CAGCTTCCTGCATGATGCTGG

Scientific, Lenexa, KS). Cultures were also supplemented with 1.5×10^5 cells/ml lethally irradiated feeder cells described elsewhere (*54*). Cells were left undisturbed for 4 days and thereafter they were disaggregated and counted daily. When the cells reached a density of 0.8×10^5 cells/ml, they were diluted 1:2 with growth media (GR10) comprised of SR10 without PHA.

Irradiations

Cultured T lymphocytes were disaggregated and seeded at a density 4×10^5 cells/ml in GR10 media. Cells were irradiated at room temperature with an HS X-ray system (AGO X-Ray Ltd., Aldermaston, UK) (output 13 mA, 250 kV peak, 0.5 Gy/min for doses above 100 mGy and 0.2 mA 4.9 mGy/min for doses up to 100 mGy). Cell cultures were maintained at 37°C after irradiation until a designated time point, and then processed according to the appropriate protocol.

For time course experiments, T lymphocytes were sham irradiated or 2 Gy X irradiated and collected 15 min, 30 min, 1, 1.5, 2, 3, 4, 5, 6 and 24 h postirradiation. For high-dose experiments, T lymphocytes were sham irradiated or irradiated with doses of 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4 and 5 Gy X rays and collected 2 or 24 h postirradiation. For low-dose experiments T lymphocytes were sham irradiated or irradiated with doses of 5, 10, 20, 30, 40, 50, 75 and 100 mGy X rays and collected 2 or 24 h postirradiation.

RNA Extraction

At each appropriate time point, cells were washed twice with cold PBS, then resuspended in 1 ml of RNA (Sigma-Aldrich Company Ltd., Gillingham, UK) and stored at −80°C until further processing. Total RNA for mRNA and lncRNA analysis was prepared using RNAqueous®-4PCR Kit (Ambion/Life Technologies Ltd., Paisley, UK). DNA contamination was removed by DNase I provided with the kit. Total RNA for miRNA analysis was prepared using the miRNeasy kit (Qiagen, Manchester, UK). DNA contamination was removed with the RNase-Free DNase Set (Qiagen, Manchester, UK). RNA quantity was assessed by Nanodrop ND1000 (Nanodrop, Wilmington, DE) and RNA quality was assessed on 1.3% agarose gel.

Gene Expression

Reverse transcriptase reactions were performed with the High Capacity cDNA Reverse Transcription Kit, (Ambion/Life Technologies Ltd., Paisley, UK) according to the manufacturer's protocol, using 700 ng of total RNA per 50 μl reaction. Real-time quantitative PCR was performed using RotorGene Q. All reactions were run in triplicate using PerfeCTa® MultiPlex qPCR SuperMix (Quanta Biosciences, Inc. Gaithersburg, MD), primer and probe sets for target genes at 300 n*M* concentration each and 1 μl of cDNA in 10 μl reaction volume. FAM, HEX, Texas Red, CY5 and ATTO680 (Eurogentec Ltd., Fawley, UK) were used as fluorochrome reporters for the hydrolysis probes analyzed in multiplexed reactions. Table 1 provides a list of the primers and probes that we designed. Cycling parameters were 2 min at 95°C, then 45 cycles of 10 s at 95°C and 60 s at 60°C. Data was collected and analyzed by RotorGene Q analysis software. Cycle threshold (Ct) values were converted to copy numbers using standard curves obtained by serial dilution of PCR-amplified DNA fragments of each gene and run with each experiment. The linear dynamic range of the standard curves covering seven orders of magnitude (from 25–48,828,125 copies per reaction) gave PCR efficiencies between 93–105% for each gene with $R^2 > 0.998$. Gene target Ct values were

normalized to the reference gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Fold-change values were obtained by normalization of irradiated samples to the appropriate controls.

miRNA Expression

MicroRNA expression experiments were performed using qScript™ microRNA Quantification System (Quanta Biosciences Inc.) according to the manufacturer's protocol. Briefly, 100 μg of total RNA was polyadenylated and reverse transcribed producing 20 μl of cDNA. Real-time quantitative PCR was performed using RotorGene Q (Qiagen). All reactions were run in triplicate using PerfeCTa® SYBR® Green SuperMix, universal primer and primer for specific miRNA at 200 n*M* concentration each and 1 μl of 50× diluted cDNA in 10 μl reaction volume. Cycling parameters were 2 min at 95°C, then 45 cycles of 10 s at 95°C and 30 s at 60°C followed by melt curve. Data were collected and analyzed by RotorGene Q analysis software. *SNORA73A* and *SNORD44* were selected by NormFinder as the most stable controls in our experimental setup.

RESULTS

Temporal Response to Ionizing Radiation

The temporal, transcriptional response to ionizing radiation was assessed in stimulated T lymphocytes (C1, C2 and AT). Cells were sham irradiated or 2 Gy X irradiated and collected at various time points ranging from 15 min up to 24 h postirradiation. We studied the expression of ten protein-coding genes, which were previously reported to be responsive to radiation either in stimulated T lymphocytes (*14*) or blood (*15*): *CDKN1A, SESN1, ATF3, MDM2, CCNB1, DDB2, FDXR, CCNG1, BBC3* (also known as *PUMA*) and *GADD45A*. The results for mRNA expression are shown in Fig. 1.

The majority of the genes investigated responded rapidly to radiation, with peak expression occurring around 2–3 h postirradiation (*CDKN1A, SESN1, ATF3, MDM2, PUMA* and *GADD45A*). Three genes, *DDB2, FDXR* and *CCNG1*, responded with slower kinetics, reaching peak expression between 5 and 24 h after exposure in the time range tested. Expression of *CCNB1* decreased rapidly after radiation exposure, but increased 24 h postirradiation. In *PUMA* and *ATF3*, two ''waves'' of transcription peaks can be seen (2 and 24 h). For all of the genes studied here, AT lymphocytes showed a lower and delayed response to radiation compared to healthy donor samples at the early time points, however, differences largely disappeared at the 24 h time point.

In addition, we investigated the response to radiation of two lncRNAs, the expression of which was reported to be altered by radiomimetic drug treatment: *TP53TG1* (*38*) and *PANDAR* (*41*), and also a FAS antisense RNA 1 (*FAS-AS1*), which is transcribed in anti-sense orientation to the FAS gene (*35*), a well known radiation-responsive transcript (*15*).

The lncRNA temporal response data are shown in Fig. 2. While *PANDAR* showed no alteration of expression after radiation exposure in the range of time points studied, *TP53TG1* demonstrated a radiation-responsive expression profile similar to *CCNG1* with a time-dependent increase in expression, however, the up-regulation stayed relatively low (maximum of 1.5× at 24 h). In contrast, *FAS-AS1* was up-regulated by up to fivefold by exposure to radiation and showed two peaks of expression: one early peak at 1.5 h and a later one around 6 h postirradiation. Similarly to expression of protein-coding genes, the *FAS-AS1* up-regulation in AT lymphocytes was delayed compared to healthy controls, however, the differences disappeared as early as 3 h postirradiation.

Next, we investigated the miRNA response to radiation exposure and we investigated the expression of 19 miRNAs, which had been highlighted as radiation responsive or were reported to be involved in the DNA damage response (DDR) network: *let-7a-5p, let-7b-5p, let-7g-5p, miR-15a-5p, miR-16-5p, miR-19b-3p, miR-21-5p, miR-27a-3p, miR-32-5p, miR-34a-5p, miR-106b-5p, miR-107, miR-125b-5p, miR-150-5p, miR-182-5p, miR-185-5p, miR-192-5p, miR-195-5p* and *miR-215-5p* (Fig. 3A). The significant modification of expression after irradiation for *miR-34a-5p* and *miR-182-5p* is shown in Fig. 3B and C, respectively. The up-regulation occurred at late time points, reaching a few folds at 24 h. Interestingly, no difference in the response to radiation between the controls and AT lymphocytes could be detected.

Dose Response to Ionizing Radiation

Dose responses were investigated for three genes presenting different temporal profiles: *CDKN1A, FDXR* and *CCNB1*. The cells were exposed to a series of doses ranging from 0.1–5 Gy and collected 2 and 24 h postirradiation. The results of the dose-response experiment are shown in Fig. 4. The shape of the dose-response curves were clearly different from samples collected at 2 and 24 h. After the 2 h time point, the data points for C1 and C2 were best fitted by a logarithmic function with strong transcriptional responses for low doses and up to 1 Gy, then reaching a plateau phase at higher doses (2–5 Gy). The transcriptional response to radiation was much weaker in AT than in C1 and C2 and interestingly, the data points for *CDKN1A* and *FDXR* were best fitted by the linear regression curve, not the logarithmic one used for C1 and C2. The dose response for *CCNB1* in the AT has a similar shape as in the controls, however, the magnitude of the repression is much lower (Fig. 4E).

The dose responses for *CDKN1A* and *FDXR* obtained from samples collected 24 h postirradiation were linear and AT could not be distinguished statistically from C1 and C2 at this time point (Fig. 4B and D, respectively). The data points for *CCNB1* were best fitted by a quadratic function with a peak of up-regulation at approximately 3 Gy. Again, the AT patient responded in the same way as healthy donors at 24 h (Fig. 4F). The T lymphocytes from the healthy donor C1 were also exposed to low doses, ranging from 5–100 mGy, results for *CDKN1A* are shown in Fig.

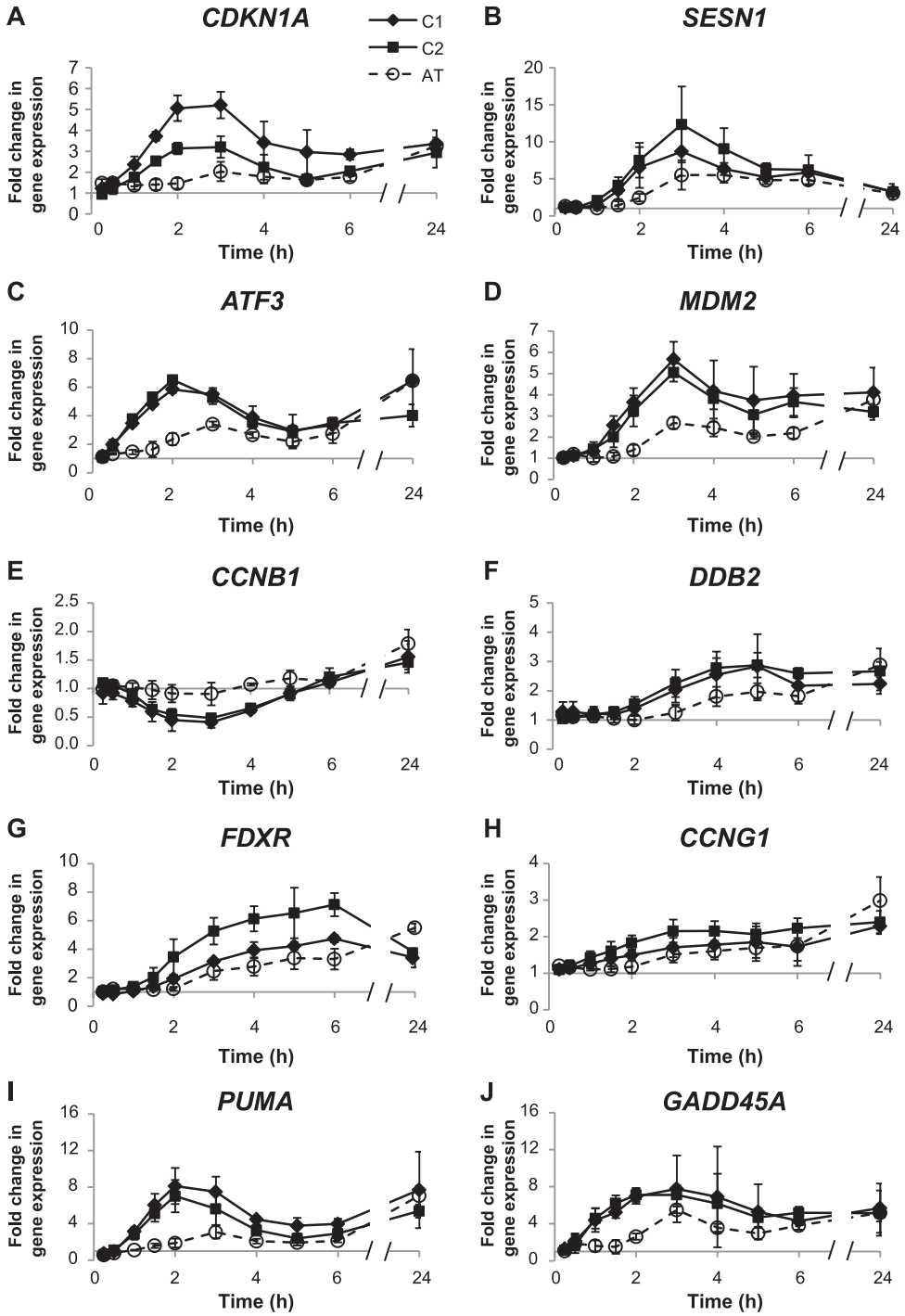

FIG. 1. Temporal expression pattern of ten protein-coding genes after radiation exposure. T lymphocytes from two healthy donors (C1: closed diamonds; C2: closed squares) and one AT patient (AT: open circles) were sham irradiated or 2 Gy X irradiated and collected at various time points ranging from 15 min up to 24 h. The expression level of genes of interest *CDKN1A* (panel A), *SESN1* (panel B), *ATF3* (panel C), *MDM2* (panel D), *CCNB1* (panel E), *DDB2* (panel F), *FDXR* (panel G), *CCNG1* (panel H), *BBC3* (*PUMA*) (panel I) and *GADD45A* (panel J) was normalized to the *HPRT1* reference gene first, then the radiation-induced fold change in expression was calculated relative to nonirradiated control. Error bars represent ± one standard deviation from three independent experiments.

4G. Interestingly, expression levels for samples at 2 h postirradiation were higher than samples collected at 24 h and the response was best fitted by a linear regression curve.

We also investigated the dose response of two lncRNAs for which we showed a modification of expression after irradiation: *TP53TG1* and *FAS-AS1* (Fig. 5). *TP53TG1* as expected from the temporal response data, only showed a

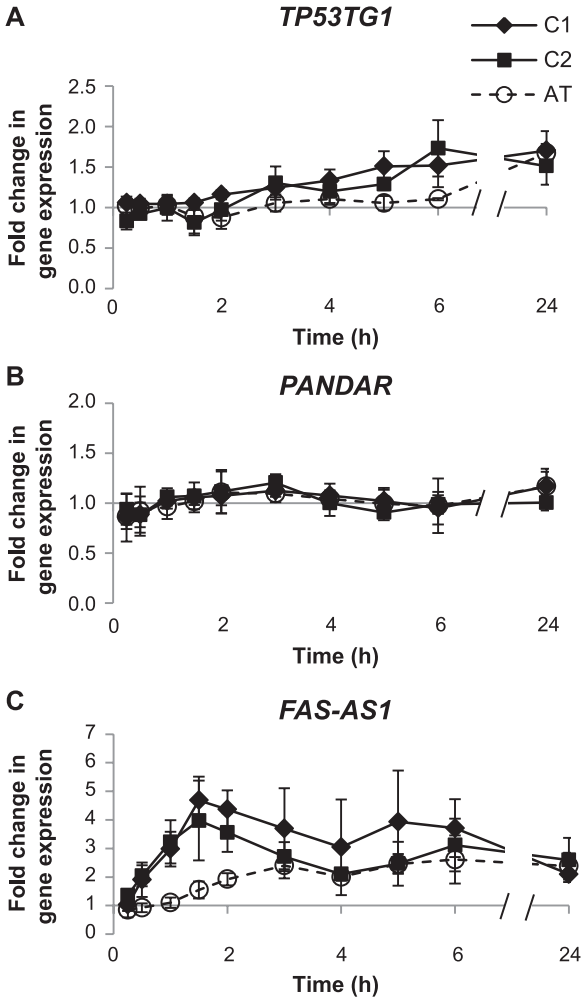

FIG. 2. Temporal expression pattern of three lncRNAs after radiation exposure. T lymphocytes from two healthy donors (C1: closed diamonds; C2: closed squares) and one AT patient (AT: open circles) were sham irradiated or 2 Gy X irradiated and collected at various time points ranging from 15 min up to 24 h. Expression levels of three lncRNAs: *TP53TG1* (panel A), *PANDAR* (panel B) and *FAS-AS1* (panel C) were normalized to the *HPRT1* reference gene first, then fold change was calculated relative to nonirradiated control. Error bars represent ± one standard deviation from three independent experiments.

marginal response to radiation at the 2 h time point, which was best fitted by quadratic regression curve (Fig. 5A); on the contrary the dose-dependent fold of change at 24 h time point was linear and reached threefold after 5 Gy. Although slightly lower, no real differences between AT and controls could be seen (Fig. 5B). The *FAS-AS1* transcript was responsive to radiation already at 2 h postirradiation and the data points for C1 and C2 were best fitted by power function regression whereas for AT it was obtained using the quadratic function. The AT showed a lower response than healthy donors, which was especially evident at lower doses (Fig. 5C). At the 24 h time point, similarly as for *CCNB1*, data points for all cells were best fitted by the quadratic function regression with a maximum of up-regulation for the highest dose tested (i.e. 5 Gy).

We then studied the dose responses for the two miRNAs which showed alteration in their expression after radiation exposure, *miR-34a-5p* and *miR-182-5p*; however, as the up-regulation was minor after 2 Gy exposure and observed only at a late time point, with no differences between the AT and the controls, we limited the experiment to C1 and C2 at the 24 h postirradiation (Fig. 6). Five doses ranging from 1–5 Gy were studied and results showed a dose-dependent up-regulation for both miRNAs with differences between C1 and C2 becoming apparent for the higher doses. This difference was already clear at the 2 Gy dose for *miR-182-5p*. Interestingly, the higher up-regulation (approximately threefold for both miRNA) with C2 cells reached a plateau phase around 5 Gy exposure, while for C1, the up-regulation was at its maximum point around 3 Gy exposure and then decreased in response to higher doses, hence showing clear differences between control cells from different donors. Data were best fitted with the quadratic function regression.

DISCUSSION

Studying gene transcription in human cells after radiation exposure provides a molecular approach for assessing radiation doses (*55*), detecting inter-individual differences in response (*56*) and aiding assessment of long-term risks (*57*). Indeed, transcription is much more complex than simply the production of transcripts of protein-coding genes and a number of miRNAs have been identified which target DDR components, e.g., *miR-100, miR-101* and *miR-421* down-regulate *ATM* expression (*58–60*), *miR-125b* and *miR-504* directly regulate *TP53* expression (*61, 62*) and *miR-605* and *miR-661* target the *MDM2* gene (*63, 64*).

The characterization of the response of noncoding RNAs to radiation exposure may be important because they have increasingly been found to be actively involved in many pathways, which may be relevant to understanding response mechanisms. Here, we have characterized the time, dose and ATM status dependency of coding and noncoding RNA expression after irradiation in stimulated human T lymphocytes.

In terms of temporal response to ionizing radiation, the majority of protein-coding genes responded to radiation very rapidly, with detectable modulation of expression as early as 30 min postirradiation for the genes *GADD45A, CDKN1A* and *ATF3* (Fig. 1). These genes play a role in cell cycle progression and checkpoints (*CDKN1A, CCNB1, CCNG1, GADD45A, SESN1*), apoptosis (*PUMA*), oxidative stress response (*SESN1, FDXR*) or TP53 stabilization (*ATF3, MDM2, CCNG1*). It is therefore not surprising that these genes respond very quickly to the insult, as many participate in the processes essential for survival and maintaining genome stability after DNA damage.

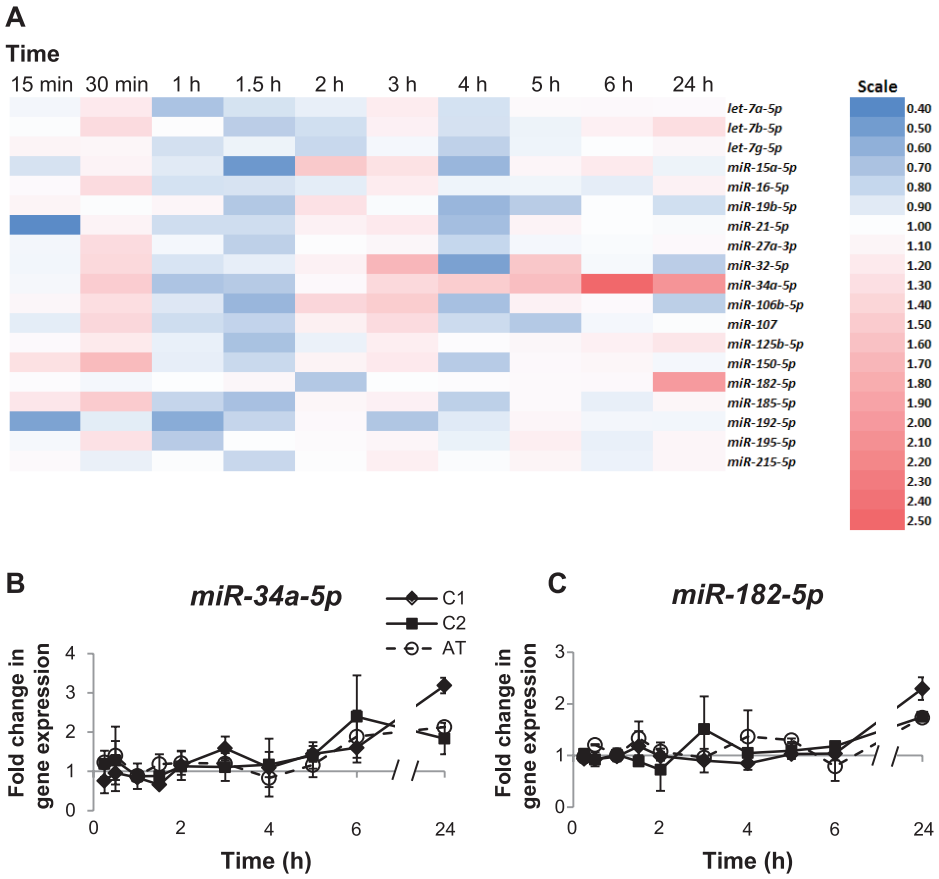

FIG. 3. Temporal expression pattern of two miRNAs after radiation exposure. Panel A shows a heat map representing time course expression profiles from 19 miRNAs in averaged C1 and C2 samples after *in vitro* 2 Gy irradiation: *let-7a-5p, let-7b-5p, let-7g-5p, miR-15a-5p, miR-16-5p, miR-19b-3p, miR-21-5p, miR-27a-3p, miR-32-5p, miR-34a-5p, miR-106b-5p, miR-107, miR-125b-5p, miR-150-5p, miR-182-5p, miR-185-5p, miR-192-5p, miR-195-5p* and *miR-215-5p*. Expression level of miRNAs was normalized to *SNORD44* and *SNORA73A* small RNA expression first, then fold change was calculated relative to nonirradiated control. The arbitrary scale is used to show up-regulated (red) and down-regulated (blue) miRNAs in irradiated samples. Temporal expression pattern of two miRNAs, *miR-34a-5p* and *miR-182-5p* is shown in panels B and C, respectively. Error bars represent ± one standard deviation from two independent experiments.

Recently, Melanson *et al*. have reported that an overwhelming majority of TP53-dependent transcripts involved in DDR, including *CDKN1A, SESN1, ATF3* and *MDM2*, are unstable, with a half-life shorter than 2 h, due to the presence of destabilizing sequences in their 3′ untranslated regions (UTRs) (*65*). The rapid turnover of TP53-regulated genes ensures plasticity of the DDR system and has one important implication for our results i.e., the fluctuations in short-lived mRNA level we observed in a time-course experiment are due to mRNA synthesis activity, since the mRNA degradation rate seems to be fast and constant. This emphasizes the importance of the time point where gene expression assessment was performed when comparing studies. The shapes of the time courses we described are likely associated with the gene-dependent mode of regulation. For example, while an early up-regulation of *PUMA* is associated with early apoptosis being triggered in T lymphocytes, the biphasic curve for *CCNB1* could be associated with cell-cycle arrest in the G_2 phase (down-regulation peak at 3 h) followed by entry into mitosis of surviving cells synchronized by radiation exposure (up-regulation peak at 24 h).

It is worth noting that Melanson *et al*. have placed *FDXR* mRNA in a stable transcript cluster with a half-life of 4–6 h, which may explain the constant increase of the *FDXR* mRNA, i.e., the mRNA is synthesized but not degraded rapidly. One could speculate that the *FDXR* transcript copy number should be less sensitive to variation with time after irradiation than the rapidly degraded genes. Indeed, *FDXR* is, in our hands, one of the best performing genes in terms of dose prediction [(*55*) and unpublished data].

We then investigated the transcriptional alterations in ncRNA expression caused by radiation exposure. Noncoding RNAs significantly outnumber protein-coding genes and their expression is very often tissue specific, therefore they are just emerging as potential biomarkers (*66, 67*). In this current study, we looked at the expression of three lncRNAs and 19 miRNAs selected from literature. One

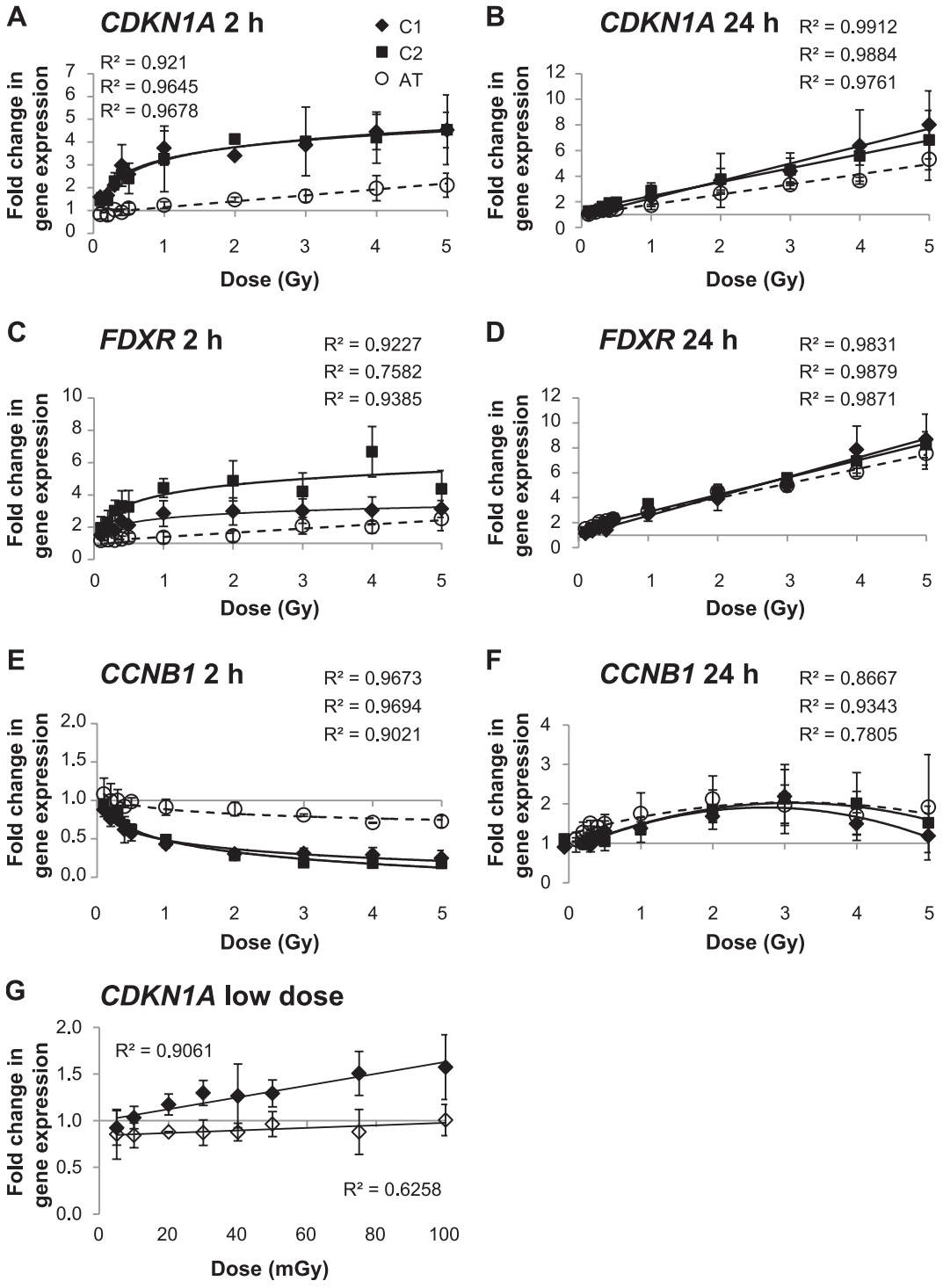

FIG. 4. Radiation dose responses of three protein-coding genes. T lymphocytes from two healthy donors (C1: closed diamonds; C2: closed squares) and one AT patient (AT: open circles) were exposed to a series of X-ray doses ranging from 0.1–5 Gy. The expression levels of three genes, *CDKN1A, FDXR* and *CCNB1*, were analyzed 2 h (panels A, C and E, respectively) and 24 h (panels B, D and F, respectively) postirradiation. Expression levels for three genes were normalized to the *HPRT1* reference gene first, then the radiation-induced fold change in expression was calculated relative to nonirradiated control. R^2 values are listed in the following order: top, C1; middle, C2; bottom, AT. Error bars represent ± one standard deviation from two independent experiments. Panel G: T lymphocytes from healthy donor C1 were exposed to radiation doses ranging from 5–100 mGy. The expression level *CDKN1A* was analyzed at 2 h (closed diamonds) and 24 h (open diamonds) postirradiation. The expression levels for three genes were normalized to the *HPRT1* reference gene first, then fold change was calculated relative to nonirradiated control. R^2 values are listed in the following order: top, 2 h; bottom, 24 h. Error bars represent ± one standard deviation from four independent experiments.

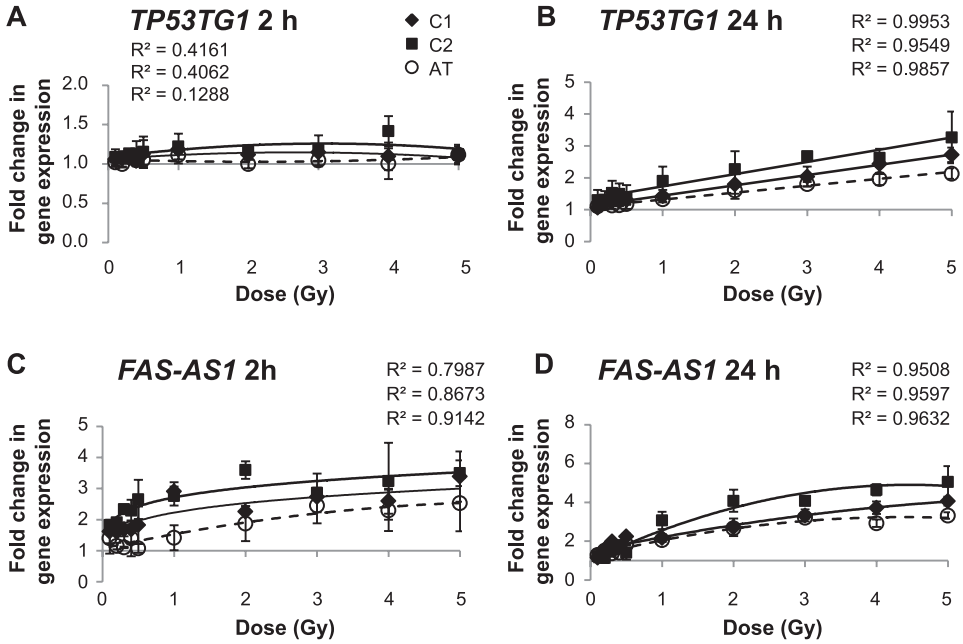

FIG. 5. Dose responses of two lncRNAs. Expression levels of two lncRNAs, *TP53TG1* and *FAS-AS1* after exposure to X-ray doses ranging from 0.1–5 Gy were analyzed 2 h (panels A, C, respectively) and 24 h (panels B, D, respectively) postirradiation. Copy numbers were normalized to the *HPRT1* reference gene first, then radiation-induced fold change in expression was calculated relative to nonirradiated control. R^2 values are listed in the following order: top, C1; middle, C2; bottom, AT. Error bars represent ± one standard deviation from two independent experiments.

lncRNA, *PANDAR*, showed no changes in expression after radiation exposure (Fig. 2B) despite the fact that it has been previously reported as up-regulated after DNA damage (*41*). Interestingly, Özgür *et al*. reported no change in *PANDAR* expression in HeLa and MCF-7 cells after irradiation or bleomycin treatment (*50*). The up-regulation of *PANDAR* after doxorubicin treatment was reported in human primary foreskin fibroblasts, which enter cell cycle arrest after DNA damage but not apoptosis. DNA damage induces a strong apoptotic response in human T lymphocytes, so it may be an evolutionary conserved, tissue-specific pattern of expression, which would explain why we did not detect an up-regulation. Tissue-specific induction of TP53 target genes in response to radiation exposure has been described before by Bouvard *et al*. (*68*) and different post-translational modifications of TP53 protein have been suggested to play a role in this process (*69*).

The second lncRNA, *TP53TG1*, showed a slight up-regulation after radiation exposure at the late time point (Fig. 2A), which was dose dependent 24 h postirradiation (Fig. 5B). *TP53TG1* is also a direct target of TP53 and has been reported to be responsive to DNA damage in the human SW480 colon cancer cell line and normal human dermal fibroblasts (*38*); again the very modest response to radiation in human T lymphocytes can be attributed to tissue specificities.

The third lncRNA investigated, *FAS-AS1*, was rapidly up-regulated by radiation exposure in C1 and C2 T lymphocytes, reaching a first peak of expression 1.5 h after exposure and a second between 5 and 6 h postirradiation. *FAS-AS1* has been identified by Yan *et al*. (*35*) as an antisense transcript of the *FAS* gene and the authors proposed that it might protect T lymphocytes from *FAS*-mediated apoptosis. We have previously shown a consistent up-regulation of *FAS* in C1 and C2 (*15*) and there is probably a fine balance between the pro- and anti-apoptotic transcripts deciding on the fate of an irradiated cell. To our knowledge, this is the first study of *FAS-AS1* being up-regulated by ionizing radiation, but we also expect or predict that there are other radiation-responsive lncRNAs awaiting discovery.

For protein-coding genes and radiation-responsive lncRNAs, the consistent feature in the AT samples, was a lower and delayed response to radiation compared to the healthy donors at the early time points; however, the difference was not detectable at the late, 24 h time point. We observed that activation of ATM downstream targets was delayed and impaired but not abrogated (Figs. 1 and 4), which while in agreement with previous studies [e.g. (*70*)], also suggests that in the absence of ATM, other pathways lead to delayed ATM downstream targets activation. Over 14 years ago, Tibbetts *et al*. suggested that another kinase, ATR, can be the major player (*71*) and subsequent studies seem to support this hypothesis (*72, 73*).

From the 19 radiation-responsive miRNAs obtained from the published literature, only two demonstrated a clear modulation of expression after radiation exposure in our experimental setup: *miR-34a-5p* and *miR-182-5p*. The

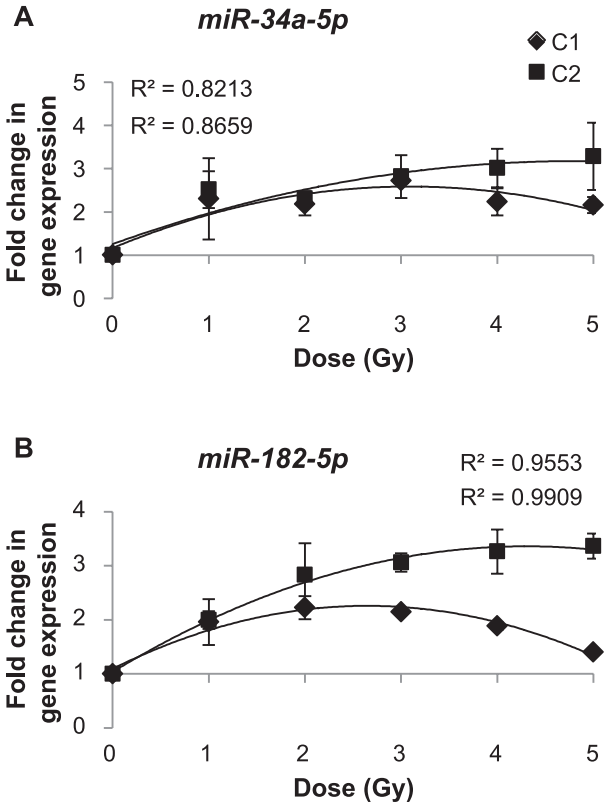

FIG. 6. Dose responses of two miRNAs. T lymphocytes from two healthy donors (C1: closed diamonds; C2: closed squares) were exposed to doses ranging from 1–5 Gy and collected 24 h postirradiation. Expression levels of *miR-34a-5p* (panel A) and *miR-182-5p* (panel B) were normalized to *SNORD44* and *SNORA73A* small RNA expression first, then the radiation-induced fold change in expression was calculated relative to nonirradiated controls. R^2 values are listed in the following order: top, C1; bottom, C2. Error bars represent ± one standard deviation from two independent experiments.

discrepancy is likely due to the fact that each study was performed with a different experimental model and with heterogeneous levels of miRNA expression. The radiation-responsive *miR-34a-5p* is a direct transcriptional target of TP53, exhibiting strong pro-apoptotic and anti-proliferative properties (*18*). The *miR-182-5p* is considered to have dual properties as an oncogene and tumor suppressor depending on the cellular context. It targets many genes positively regulating DDR but also cyclin-dependent kinase 6 (CDK6), which phosphorylates retinoblastoma 1 protein (RB1) and consequently promotes cell cycle progression (*74*). Both miRNAs were up-regulated at the latest 24 h time point and we could not detect any differences between healthy controls and the AT.

CCNB1 is a main cyclin active during G_2/M phase of the cell cycle and together with cyclin-dependent kinase 1 (CDK1) it forms a maturation-promoting factor that is necessary for entry into mitosis. Therefore *CCNB1* expression is under tight control, since entering mitosis with unrepaired DNA damage is potentially very dangerous to cells (*75*). In this study, *CCNB1* expression in C1/C2 is significantly repressed by doses as low as 0.4 Gy 2 h postirradiation; previous studies have shown that while G_2/M arrest is ATM dependent at an early time point postirradiation (*76*), at later time points it becomes ATR dependent as S-phase cells progress into G_2 phase (*77, 78*). Our data obtained at the transcriptional level fit very well with these previous findings, thus validating the transcriptional responses analyses to provide relevant information about DNA damage-associated molecular mechanisms.

For biological dosimetry purposes, *TP53TG1* appears to be a suitable candidate since, although it is not modified by radiation 2 h post exposure, a clear linear dose response was seen for all controls and AT 24 h post exposure. On the contrary, *FAS-AS1* might not be suitable as an accurate biomarker of exposure since its up-regulation reached a plateau at around 1 Gy 2 h postirradiation, and is not linear (best fitted by a polynomial regression curve) at 24 h. Nevertheless, the ATM-dependent transcriptional activation we have described here is of great interest, and further research is required to discover its role in the DDR after radiation exposure. We have also confirmed the radiation responsiveness of two miRNAs in cultured T lymphocytes and they might be of interest as exposure biomarkers if their expression pattern *in vivo* in blood is similar. It is very likely that after *in vivo* irradiation, the blood will contain other radiation-responsive miRNAs in exosomes. For example, Jacob *et al*. (*25*) identified *miR-150* as a sensitive biomarker of *in vivo* exposure in mouse serum.

We have shown that the transcriptional response of human T lymphocytes can be accurately detected even with low-dose radiation (5–100 mGy) at 2 h post exposure. *CDKN1A* showed a linear response to radiation at both time points, and our data demonstrate that at the transcriptional level cells can detect very low doses of radiation (10–20 mGy) and the genes responding to low doses could be potentially used as biomarkers of low-dose exposure.

In summary, our data indicate that studying gene expression at early time points can highlight individuals with AT deficiency and potential associated sensitivity to ionizing radiation. We have previously demonstrated that monitoring expression of TP53 downstream targets in response to radiation can be used as a surrogate assay for assessing ATM/CHK2/TP53 pathway activity and individual cancer risk (*57*) when analyzed at an early time point (i.e. 2 h). The results presented here suggest that it is best to use a 24 h time point for biodosimetry purposes, as the dose response becomes linear and inter-individual differences in radiation sensitivity (at least for ATM/CHK2/TP53 pathway) do not confound the response. This study provides evidence that radiation exposure elicits dose- and time-dependent changes in the expression of coding and noncoding RNA that are influenced by the genetic background. Furthermore, it suggests that noncoding RNAs may be a potentially rich

source of biomarkers for radiation exposure, predisposition or long-term effects.

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