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Aberrant overexpression of miR-421 downregulates ATM and leads to a pronounced DSB repair defect and clinical hypersensitivity in SKX squamous cell carcinoma

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ABSTRACT

Background: Cellular and clinical sensitivity to ionizing radiation (IR) is determined by DNA double-strand breaks (DSB) repair. Here, we investigate the molecular mechanism underlying the extreme response of a head and neck tumor case (SKX) to standard radiotherapy.
Methods: Immunofluorescence (IF) was used for the assessment of DSB repair, Western blot and real-time PCR for protein and mRNA expression, respectively.
Results: SKX cells exhibited a pronounced radiosensitivity associated with numerous residual γ-H2AX foci after IR. This was not associated with lacking canonical repair proteins. SKX cells did not express any ATM protein. Accordingly, immunoblotting revealed no ATM kinase activity toward substrates such as p-SMC1, p-CHK2 and p-KAP1. Sequencing of all 66 exons of ATM showed no mutation. ATM mRNA level was moderately reduced, which could be reverted by 5′-Aza-C treatment but without restoring protein levels. Importantly, we demonstrated a post-transcriptional regulation in SKX cells via 6-fold enhanced levels of miR-421, which targets the 3′-UTR of ATM mRNA. Transfection of SKX cells with either anti-miR-421 inhibitor or a microRNA-insensitive ATM vector recovered ATM expression and abrogated the hyper-radiosensitivity.
Conclusion: This is the first report describing microRNA-mediated down-regulation of ATM leading to clinically manifest tumor radiosensitivity.
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DSB-mediated tumor cell killing remains the mainstay of radiotherapy (RT). Indeed, tumor cells vary in their response to IR depending on tumor origin, histology and most importantly, DSB repair capacity [1]. There are two main pathways of DSB repair, nonhomologous end joining (NHEJ) and homology-directed repair (HR). Any defect in one of the canonical repair proteins of NHEJ leads to clearly reduced DSB repair capacity and enhanced cellular radiosensitivity [2]. Furthermore, alteration in the upstream DNA damage response (DDR) signaling may confer repair defects and hence enhances cellular radiosensitivity. Among them, defects in ataxia-telangiectasia mutated (ATM) are most prominent. ATM transduces a DSB damage signal to downstream effectors by phosphorylating critical proteins including itself, H2AX, SMC1, KAP1, and ChK2 in order to generate an appropriate signal for the cells to respond [3]. Variations in the expression of NHEJ and DDR genes were found to have a strong impact on tumor response to RT [4–7]. However, a clear defect i.e. mutation in one of these genes in human malignancies was rarely reported, indicating that genetic alterations per se cannot account for these variations. This raises a possibility of post-transcriptional regulation, of which microRNAs (miRNAs) are considered among the largest class of gene regulators. miRNAs are 18–22 non-coding RNA nucleotides that regulate the gene expression by binding to the 3′-untranslated region (3′-UTR) of the mRNA of specific genes through sequence complementary. This interaction can trigger the inhibition of translation or degradation of the targeted transcript [8–10]. Of note, aberrant expression of miRNAs was recently reported in different tumors [7,11–15].

Understanding of the mechanism and biochemical details of the DSB response and repair, especially in tumors, is required, in order to predict response to IR and eventually improve RT outcome. In this context analysis of tumors with extreme radiosensitivity is

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considered to be especially informative, since they may help to identify specific defects which can be used either as a marker or as a new target. This strategy was recently successfully used to identify tumors deficient in BRCA1/2 for a PARP1 directed target therapy [16–18].

We have recently characterized a squamous cell carcinoma cell line SKX showing an extreme radiosensitivity associated with a clear DSB repair defect [19]. In the current study, we identified the underlying molecular mechanism behind this extreme response to IR. We suggest that this was causally associated with overexpression in miR-421 that inhibits ATM translation, resulting in an extreme cellular ATM deficiency and clinical radiosensitivity. These findings suggest that miR421 may be a prediction biomarker of clinical radiotherapy.

Materials and methods

Cells and transfections

The SKX HNSCC cell line was established from a biopsy obtained from bulky oral carcinoma (T4, N2, M0) of an 83-year old patient at the University of l Hamburg in 1991 who clinically responded remarkably well to conventional neo-adjuvant radiotherapy (two opposing field with 60 Gy in 2 Gy-fractions, plus a 10 Gy boost to the tumor) [20] without further need for surgery. All HNSCC tumor cell lines (UTSCC5, 8, 14, and 15, SCC45, SAS, Cal33, XF345, HCS4, SAT, A431, FaDu, SKX) as well as HeLa, and H1299 cells were cultured in DMEM supplied with 10% FBS and 1% streptomycin/penicillin under the optimum culture conditions (37 °C, 10% CO_2). The pre-miR421 precursor, pre-miRNA control precursor, Anti-miR negative control 1, and Anti-miR421 inhibitor were purchased from Applied Biosystems. All transfections were done with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ATM expression plasmid pMAT1 (a kind gift from M. Lavin) was transfected into cells using FuGENE HD transfections reagent (Roche) according to manufacturer's protocol.

Protein extraction and Western blot

Whole cell lysates were isolated using Laemmli buffer [21] and nuclear lysates were isolated with Subcellular Protein Fractionation Kit (Thermo Scientific). Western blot was performed according to the standard protocol. Antibodies used: rabbit anti-ATM, mouse anti-pS1981-ATM, mouse anti-Chk2, rabbit anti-pT68-Chk2 (Cell Signaling), mouse anti-pS957-SMC1 (Rockland), rabbit anti-pS966-SMC1 (Bethyl Laboratories), mouse anti-SMC1 (R&D systems), rabbit anti-KAP1 and rabbit anti-pS824-KAP1 (Bethyl Laboratories) as well as mouse anti-β-actin (Sigma).

Immunofluorescence

Immunofluorescence staining of γ-H2AX and ATM was performed as previously described [22]. Antibodies used: mouse monoclonal anti-phospho-S139-H2AX antibody (Cell Signaling), rabbit monoclonal anti-ATM (Epitomics), anti-mouse Alexafluor594 (Invitrogen), anti-rabbit fluorescein (Amersham). The nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI, 10 ng/ml). Immuno-fluorescence was observed with the Zeiss AxioObserver.Z1 microscope.

DNA-PK activity

The DNA-PK activity was determined by a pull-down assay as described before [23]. All assays were performed multiple times with at least two different extracts.

Luciferase Reporter Assay

Cells were co-transfected with *Renilla* luciferase plasmid that harbors the full-length 3′UTR of *ATM* cloned downstream the luciferase open reading frame and either pre-miR-421 precursor (50 nM), pre-miR-control (50 nM), Anti-miR-421 inhibitor (50 nM) or Anti-miR negative control (50 nM). A firefly luciferase plasmid pGL3 control was co-transfected to normalize to the transfection efficiency. Cells were lysed at 24 h or 48 h after transfection, and luciferase activity was measured with the Dual-Glo assay (Promega). Data are presented as the mean ± SEM from three samples per data point.

RNA extraction and real-time quantitative PCR

Total miRNAs from cultured cells was extracted by the TaqMan MicroRNA Cells-to-CT Kit (Applied Biosystems). Taq-Man microRNA expression assays (Applied Biosystems) were used to quantify mature miR-421 expression according to the manufacturer's protocol. Human 18S rRNA was used as internal control for miR-421 expression. *ATM* and *MYCN* mRNA quantifications were measured by real-time PCR on a Rotogene 6000 (Corbett) after extraction of total RNA using Trizol reagent (Invitrogen). *ACTB* mRNA was used as an internal control to normalize *ATM* mRNA level. Real-time PCR was performed in triplicates and quantified using a $2^{-\Delta\Delta C}{}_T$ method [24].

Clonogenic survival assay

Cells were plated at 400 cells per T25 flask in triplicate and then incubated for 2–4 h to allow attachment. Cells were X-irradiated (RS225 research system, GLUMAY MEDICAL, UK at 200 kV, 15 mA) and maintained for 2–3 weeks before staining with 1% crystal violet. Colonies containing more than 50 cells were counted as survivors (automated Colcount Mammalian Cell Colony Counter Oxford Optronix Ltd., UK).

Genomic sequencing

All exons of the *ATM* gene were then subjected to PCR using previously described primer pairs and PCR conditions PCR products were purified by polyethyleneglycol precipitation and subjected to Sanger sequencing using BigDye chemistry (Applied Biosystems). Reaction products were separated by capillary gel electrophoresis on an Avant 3100 Genetic Analyser (Applied Biosystems), and electropherograms obtained using Sequence Analysis Software were compared against the *ATM* reference sequence as deposited in GenBank (www.ncbi.nlm.nih.gov/; NG009830.1).

5′-Aza-2′-deoxycytidine treatment

For 5′-Aza-C treatment, SKX and FaDu cells were plated in 6-well plates and were incubated either without or in the presence of 0.1 or 0.2 μM 5′-aza-2′-deoxycytidine (Sigma), respectively. Cell culture medium supplemented with 5′-azaC was changed daily, and cells were then harvested after four rounds of 5′-azaC treatment.

Statistical analysis

All statistical analyses have been performed using Graphpad Prism version 5.04. Data are represented as means ± SEM of at least three different experiments. Statistical significance was determined with unpaired Student *t* tests. *P* values less than .05 were considered statistically significant.

Results

SKX cells show repair deficiency but express normal levels of DSB repair proteins

Confirming our previous observations [19,20], SKX cells were extremely radiosensitive compared to a panel of HNSCC (Fig. 1A), representing – to the best of our knowledge – the most sensitive HNSCC in the literature. The SKX was functionally compared to the FaDu cell line which is of average radiosensitivity (Fig. 1A). SKX cells displayed about 3-fold more unrepaired DSBs than FaDu cells 24 h after 4 Gy (Fig. 1B) confirming the significant DSB repair defect previously described [19,20]. In order to verify the mechanism of this DSB deficiency, we firstly investigated the expression levels of classical nonhomologous end joining (NHEJ) proteins such as KU70, KU80, DNA-PKcs, XRCC4, Ligase IV, and also of RAD51. No significant differences were found comparing SKX to FaDu cells (Fig. 1C and Supplementary Fig. 1A). Functional analysis of DNA-PKcs even showed 2-fold higher kinase activity in SKX compared to FaDu cells (Supplementary Fig. 1B). Together, we confirmed the hypersensitivity and DSB repair defect in SKX.

SKX cells are ATM-deficient

Interestingly, the time course for DSB repair measured by persistence of γ-H2AX foci at 24 h after 4 Gy (Fig. 1B) revealed about 15% unrepaired breaks in SKX cells which is exactly the value reported for AT cells [25]. Strikingly, we could not detect any traces of ATM protein, neither in whole cell nor in nuclear extract of SKX cells (Fig. 1D, fractionation markers are shown in Supplementary Fig. 1D), revealing a pronounced ATM deficiency. Furthermore, the phosphorylation profile of ATM downstream targets 30 min after IR revealed a lack of ATM auto-phosphorylation (p-ATM-S1981), of p-SMC1 (S957 and S966), p-Chk2 (T68), and p-KAP1 (S824) in SKX cells (Fig. 1D and Supplementary Fig. 1C), while these target proteins were normally phosphorylated in FaDu, HeLa or MCF-7 cells. To further causally associate the ATM deficiency with the underlying cellular radiosensitivity, cells were treated with 10 μM of the ATM-specific inhibitor KU55933 and analyzed for IR sensitivity. While FaDu cells were substantially sensitized to IR to levels of SKX, no further radiosensitization was seen for SKX cells (Fig. 1E). Together, these data strongly suggest that SKX cells are ATM-deficient and that this defect is related to the extreme radiosensitivity of SKX cells.

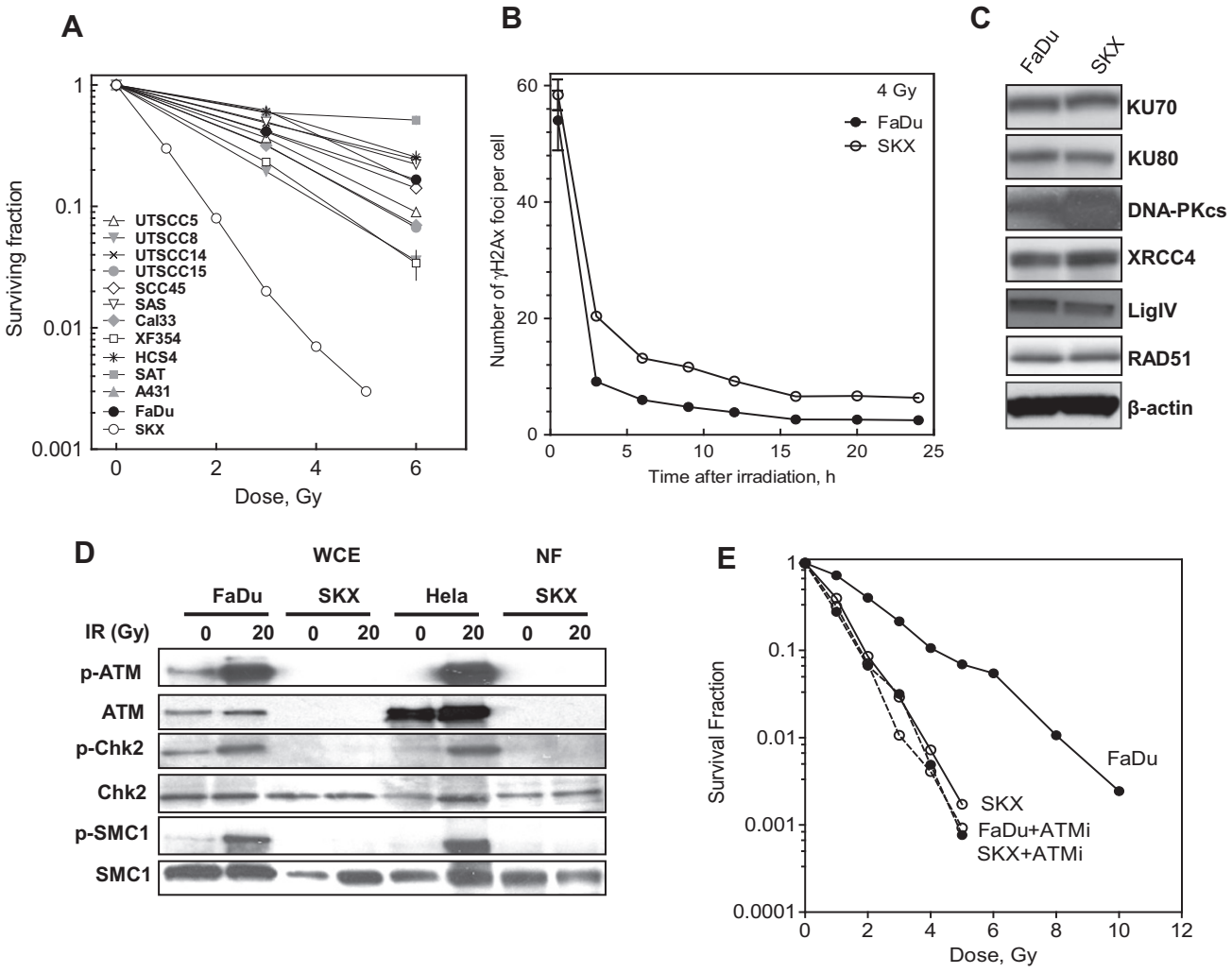

Fig. 1. SKX cells are ATM-deficient which render them extreme radiosensitive. (A) 13 HNSCC tumor cell lines were irradiated at the indicated doses and survival rates were measured after 3 weeks. (B) Immunofluorescence analysis of γ-H2AX foci 24 h post X-irradiation (4 Gy) of both FaDu and SKX cells. (C) Western blot analysis of DSB repair proteins expression (KU70, KU80, DNA-PKcs, XRCC4, LIGIV and RAD5) in SKX and FaDu cells. β-actin was used as a loading control. (D) Western blot analysis of ATM expression in SKX (in whole and nuclear extracts) compared to FaDu and HeLa cells. 30 min after 20 Gy, SKX cells showed an impaired ATM damage signaling indicated by the absence of p-ATM, p-SMC1, and p-CHK2. Unphosphorylated proteins (SMC1 and CHK2) served as loading controls. (E) Survival rate after inhibition of ATM using a specific inhibitor KU55933 (10 μM). Survival rate was measured as in A. Shown are mean ± SEM values of 3 experiments.

ATM deficiency in SKX cells is post-transcriptional

We next sought to identify the underlying mechanism of the ATM defect in SKX cells. DNA Sequencing of all coding exons and the large 3′-UTR of *ATM* revealed no somatic mutations excluding the possibility that the ATM-deficiency in SKX cells is explained at the DNA level. Next, we investigated the integrity and quantity of the *ATM* mRNA. We employed nine partially overlapping primer pairs (see Supplementary Table 1) widely distributed over the *ATM* transcript (Fig. 2A). Reverse transcription and successful PCR amplification of all expected fragments demonstrated the physical presence and integrity of *ATM* mRNA in SKX cells (Fig. 2B). Quantitative real-time PCR showed a 2-fold reduced level of *ATM* mRNA in SKX compared to FaDu cells (Fig. 2C). This prompted us to investigate ATM promoter hyper-methylation, which had previously been reported in about 20% of HNSCC [5]. To this end, we reduced DNA methylation by growing the cells for several rounds in 5′-Aza-2′-deoxycytidine (5′-Aza-C) which in fact raised the mRNA levels by two-fold (Fig. 2C). Although mRNA level was now comparable to that of untreated FaDu cells, we still could not detect any ATM protein in SKX cells by immunoblotting (Fig. 2D). These data demonstrate that the lack of ATM protein in SKX cells is not due to low mRNA level but rather due to a post-transcriptional regulation.

ATM 3′-UTR is targeted by microRNAs in SKX cells

To explore the possibility that ATM might be regulated by miRNAs, we transfected both FaDu and SKX cells with the luciferase reporter (pRL-3′-UTR) that contains the 3′-UTR of *ATM* downstream of the luciferase open reading frame [26]. Luciferase activity measured 48 h-post transfection was significantly reduced (76%) in SKX compared to FaDu cells (Fig. 3D). These data suggest that ATM expression is under tight control of its 3′-UTR, most likely mediated through microRNAs.

miR-421 is over-expressed in SKX cells and targets the 3′-UTR of ATM

Hu and colleagues have recently reported that miR-421 might regulate ATM expression through binding a sequence within the 3′-UTR of ATM [26], making this particular miRNA a good candidate as the potential causal signal for radiosensitivity in SKX cells. First, we examined the effect of miR-421 on 3′-UTR of ATM by co-transfecting the pRL-3′-UTR luciferase reporter together with pre-miR-421 precursor into both HeLa and FaDu cells. Luciferase activity was specifically suppressed in both HeLa (30%) and FaDu (40%) cell lines in the presence of miR-421 (Fig. 3A), revealing that miR-421 is a relevant negative regulator of ATM. Next, the endogenous miR-421 level in SKX cells was quantified by real-time PCR. Strikingly, we found a substantial over-expression (6-fold) of miR-421 in SKX compared to FaDu cells (Fig. 3B). Direct sequencing of both the genomic locus of miR-421 (plus adjacent miR374b) as well as its binding site in the ATM 3′-UTR did not reveal any alteration. However, *MYCN*, a positive regulator of miR-421 [26], was found to be 15-fold enhanced in SKX cells compared to FaDu (Fig. 3C). Altogether, these data illustrate that the over-expression of miR-421 in SKX cells, probably due to increased *MYCN* expression [26], inhibits ATM protein expression by targeting its 3′-UTR.

Inhibition of miR-421 rescues SKX cells from hyper-radiosensitivity by re-expression of ATM

Next, we challenged miR-421 in SKX cells by transfecting an anti-miR-421 inhibitor that competitively binds endogenous miR-421 and measured the effect on ATM 3′-UTR-luciferase activity. Anti-miR-421 in fact increased luciferase activity by three fold in SKX cells (Fig. 3D) compared to only a minor increase in FaDu cells. Importantly, the miR-421 inhibitor led to re-expression of ATM-protein in SKX cells (Fig. 4A) which consequently resulted in a substantial reversion of the hyper-radiosensitive phenotype (Fig. 4B). In a complementary approach to verify the miRNA-mediated

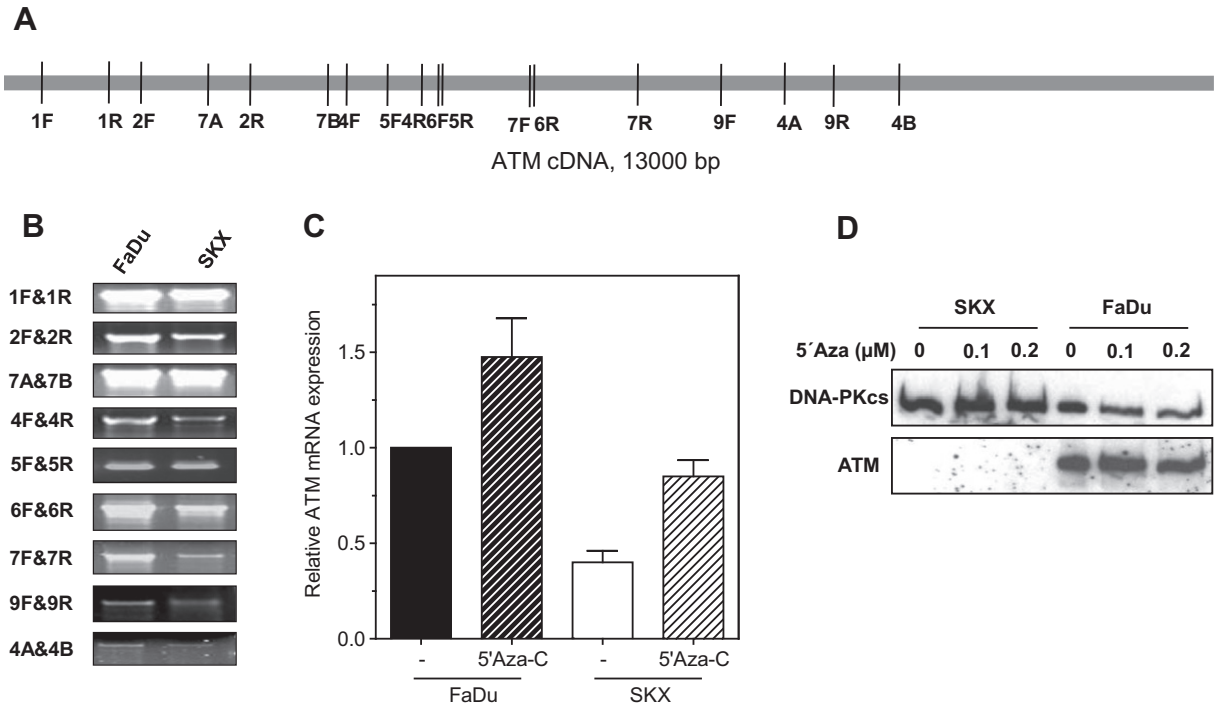

Fig. 2. ATM deficiency in SKX cells is post-transcriptional. (A) To-scale schematic representation of *ATM* transcript showing the positions of the primers used in the current study. (B) RT-PCR using the above shown overlapped primers showing all expected amplified fragments in both FaDu and SKX cells. (C) Real-time PCR analysis showing the relative expression of *ATM* mRNA in SKX and FaDu cells. (D) Real-time PCR analysis showing the relative *ATM* mRNA expression after 5′-Aza-2′-deoxycytidine treatment. Shown are mean ± SEM values of 3 experiments.

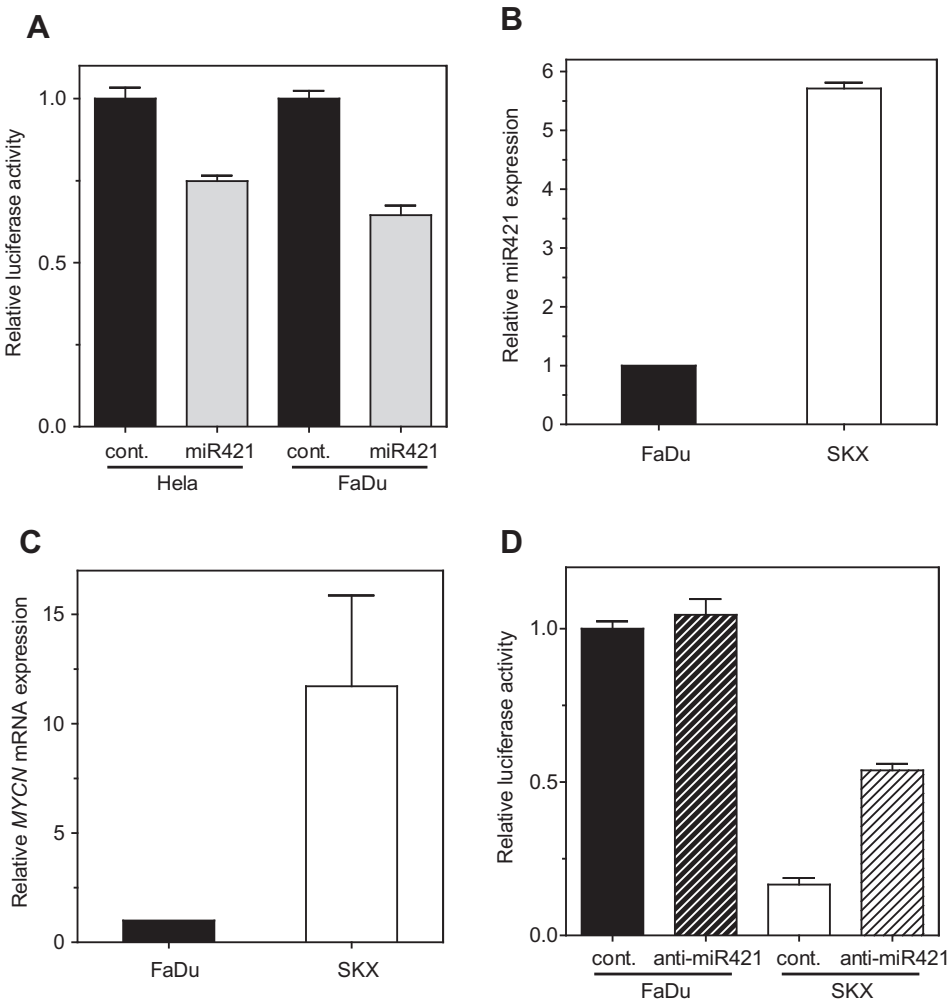

Fig. 3. miR-421 is over-expressed in SKX cells. (A) Both control luciferase (pGL3) and luciferase vector that contains ATM 3′-UTR (pRL 3′-UTR) were co-transfected with pre-miR negative control (cont.) or pre-miR-421 precursor (miR-421) into HeLa and FaDu cells and luciferase activity was measured 48 h later. (B) Relative miR-421 expression in FaDu and SKX cells. (C) Real-time PCR analysis showing the relative expression of *MYCN* mRNA in both FaDu and SKX cells. Shown are the mean ± SEM values of 3 experiments. (D) The luciferase activity was measured as in A after transfecting SKX cells with either Anti-miRNA negative control (cont.) or inhibitor anti-miR-421. Shown are mean ± SEM values of 3 experiments.

regulation of ATM, we transfected SKX cells with a microRNA-insensitive ATM-expression vector (lacking the endogenous 3′-UTR) (pMAT1 [27]), and measured the persistence of γ-H2AX foci 24 h post-IR (3 Gy) by immune-fluorescence microscopy (Fig. 4C, upper panel). 24 h after IR, individual SKX cells that express the ATM transgene had significantly lower amounts of residual DSBs ($p < 0.0001$) compared to those that do not (Fig. 4C, lower panel). Notably, DSB repair efficiency of ATM-complemented SKX cells is comparable to that of FaDu cells (Fig. 4C, see columns 4 and 6). This approach also rescued the SKX cellular extreme radiosensitivity of SKX cells (Fig. 4D). Collectively, these results proved that suppression of ATM expression in SKX cells and hence their extreme radiosensitivity is miR-421-mediated.

In order to verify the hypothesis that miR-421 over-expression sensitizes tumor cells to IR, we transfected HeLa and H1299 cells with pre-miR-421 precursor to transiently overexpress this microRNA. Indeed, miR-421 overexpression downregulates ATM expression which resulted in significant radiosensitization of HeLa and H1299 cells (Fig. 5A and B).

Discussion

Here we decipher the molecular mechanism underlying the extreme response of a human head and neck tumor (SKX) to radiation treatment [19,20,28]. Both cells and xenografts showed extreme hypersensitivity to IR and a delayed and incomplete DSB repair (Fig. 1A, B and [19,20]). On one hand, the lack of an obvious NHEJ defect (Fig. 1C and Supplementary Fig. 1A and B) and, on the other hand, the striking similarities in the DSB repair kinetics of SKX and AT cells (Fig. 1B) led us to suspect an ATM defect in these tumor cells despite any clinical hint of a germ line ATM mutation. We confirmed the ATM-deficient phenotype by two lines of evidence: (1) SKX cells express no ATM protein and show an impaired ATM signaling after IR, as indicated by the absence of phosphorylation of all tested downstream targets (Fig. 1D). (2) ATM inhibition did not further sensitize SKX cells to IR as it did for FaDu cells (Fig. 1E).

The ATM-deficiency of SKX cells was not due to exon or splicing mutations in *ATM* gene. Although SKX cells express slightly lower *ATM* mRNA (Fig. 2C) increasing the amount of the *ATM* transcript by treatment with demethylating 5-Aza-C (Fig. 2C) did not result in re-expression of ATM protein (Fig. 2D). Instead, we present evidence for a post-transcriptional regulation of ATM expression mediated by a microRNA that has hitherto not been appreciated in a clinical setting. MiR-421 was shown to be 6-fold up-regulated (Fig. 3B) and to efficiently target the ATM 3′-UTR in SKX cells (Fig. 3A and [26]). We also reported an over-expression of *MYCN*, a positive regulator of miR-421 [26], in SKX cells (Fig. 3C) which could well provide the mechanism for miR-421 up-regulation.

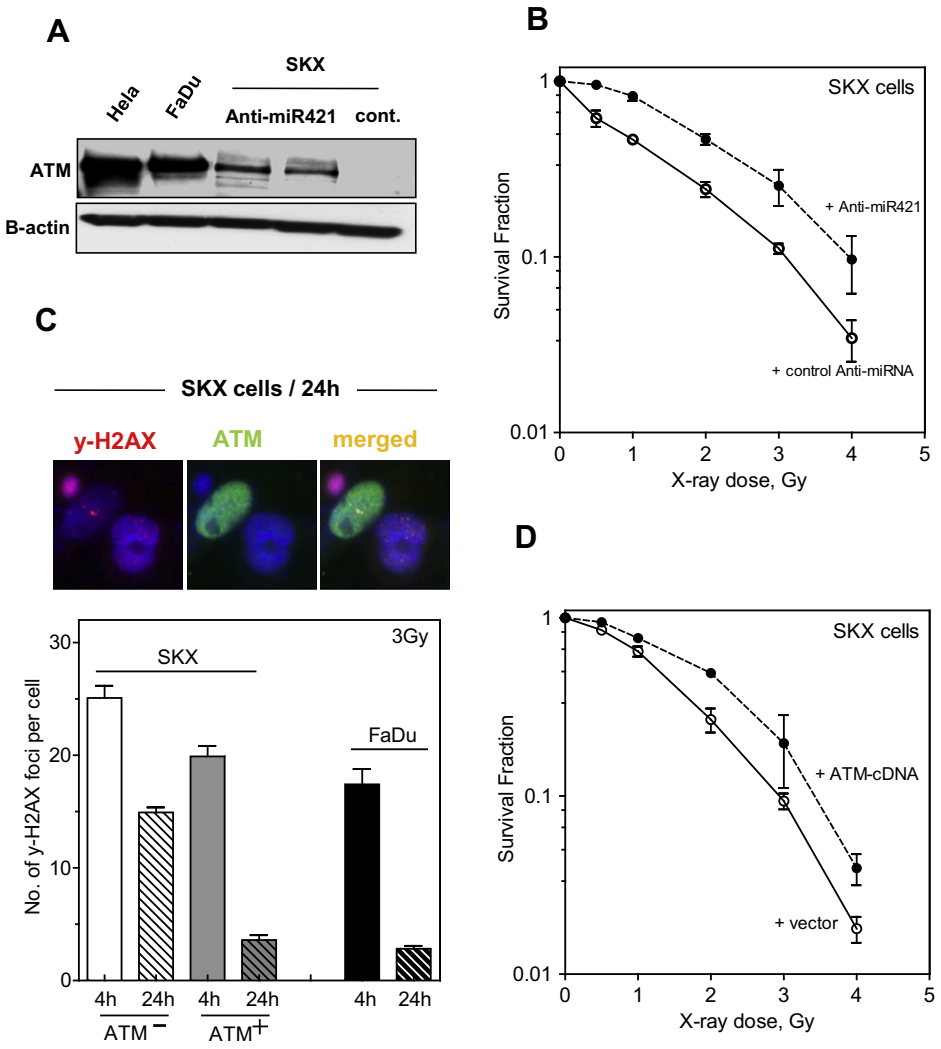

Fig. 4. Inhibition of miR-421 decreases the extreme radiosensitivity in SKX cells. (A) Western blot analysis of ATM in SKX cells after miR-421 inhibition by treatment with Anti-miR-421 inhibitor (lanes 3&4). (B) Cells were first transfected by either Anti-miR-421 or Anti-microRNA negative control, incubated for 24 h and survival rate was measured as in Fig. 1. (C) Cells were transfected with *ATM* cDNA lacking the 3′-UTR and 24 h-post transfection cells were irradiated with 3 Gy. 4 h and 24 h-post irradiation, γ-H2AX foci were measured in ATM-positive or negative SKX cells. (D) Survival rate was measured in SKX cells after transfection with microRNA-insensitive ATM expressing vector. Shown are mean ± SEM values of 3 experiments.

Inhibition of the miR-421 or overexpression of a miRNA-insensitive *ATM* cDNA (3′-UTR deleted) (Fig. 4) in SKX cells rescued DSB repair capacity and significantly increased their radioresistance. Furthermore, ectopic expression of miR-421 downregulated ATM expression and sensitized both HeLa and H1299 cells to IR (Fig. 5A and B). On the basis of these findings, we propose a model for the extreme response of the SKX patient to radiotherapy (Fig. 6). The tumor cells of this patient exhibit an up-regulation in miR-421 expression, due to *MYCN* over-expression, which impeded DSB repair capacity by inhibiting ATM expression, rendering them extremely radiosensitive.

ATM-mediated DNA damage response (DDR) is a physiological barrier which is attenuated during carcinogenesis [29–33]. Indeed, ATM had been reported to be down-regulated in a variety of cancers without any evidence for gene mutations [34–36].The present study now substantially expands our understanding of how this can be achieved, namely by up-regulation of miR-421 eventually in concert with others, such as miR100 [37] and miR101 [38]) that lead to down-regulation of ATM. In support of this notion, it was shown that miR-421 is up-regulated during physiological development of B cells [39] which undergo V(D)J and class switch recombination [40] during plasma cell maturation. DSB are deliberately introduced during these recombination processes which in turn potentially induce an anti-proliferative DDR orchestrated by ATM. Up-regulation of miR-421 likely serves to counterbalance this process keeping the ATM signal below a physiological threshold. Recapitulating this genetic program temporarily or permanently – as in our case – could enable individual mutated cells to overcome the physiological barrier on their road toward cancer. Although the exact mechanism of up-regulation of anti-ATM microRNA warrants further elucidation, general evidence has be presented that (i) a mutation of microRNAs or their regulators (e.g. *MYCN* gene amplification) might result in over-expression of the respective microRNAs, or (ii) a mutation in the 3′-UTR might enhance its binding affinity to microRNAs, leading to down-regulation of the target mRNA [41]. Because direct sequencing of the 3′-UTR has not revealed a mutation in the ATM cDNA sequence of SKX and the miR-421 binding sequence at c.*969–991 was wild type in this cell line, we consider it likely that upstream regulators are responsible for miR-421 over-expression. Indeed, the *MYCN* oncogene that we find strongly expressed in the SKX cells has previously been proposed as an important transcriptional activator of miR-421 [26].

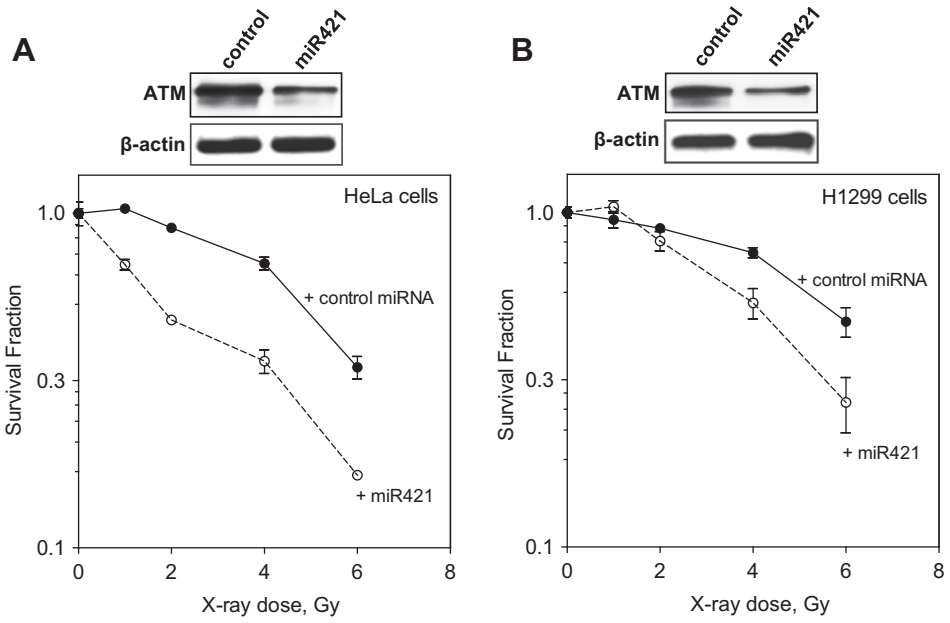

Fig. 5. miR-421 sensitizes tumor cells to IR. (A) Western Blot for ATM expression (upper panel) and survival rate (lower panel) in HeLa cells after transfection with either pre-miR negative control (+control) or pre-miR-421 precursor (+miR-421). (B) The same as in A, but for H1299 cells. Shown are mean ± SEM values of 3 experiments.

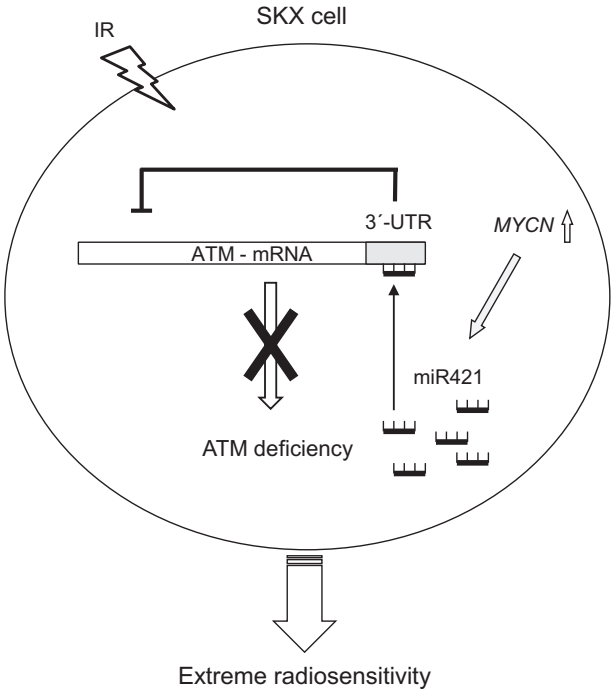

Fig. 6. A model for the extreme radiosensitivity in SKX patient. Our study suggests that SKX patient showed an extreme response to radiotherapy because miR-421 is over-expressed in his tumor cells, which is associated with *MYCN* overexpression. This impeded DSB repair capacity by inhibiting ATM expression rendering them extremely radiosensitive.

Altogether, this study provides the first proof of principle evidence for the involvement of miRNA (i.e. miR-421) as an important mechanism for ATM down-regulation in a human malignancy that leads to clinically manifest radiosensitivity in an individual tumor. It is not known yet how frequent miR-421 up-regulation occurs and whether it regularly accompanies *MYCN* amplifications reported in other malignancies [26]. Since miR-421 overexpression confers enhanced sensitivity and, hence, tumor control, it might have been overseen in the past clinical practice. However, our results suggest that miR-421 expression levels could be of prognostic value in predicting the outcome of radiotherapy. Furthermore, the demonstration of the key-role of miR-421 for inhibition of ATM expression and hence the extreme radiosensitivity in SKX cells provides the ground for the development of miR-421 analogs as therapeutic molecules to sensitize tumors to IR (see Fig. 5).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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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.radonc.2012.10.020.

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