

Dose-dependent expression of extracellular microRNAs in HCT116 colorectal cancer cells exposed to high-dose-rate ionising radiation

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Abstract. Biomarkers of tumour response to radiotherapy may help optimise cancer treatment. The aim of the present study was to identify changes in extracellular microRNAs (miRNAs) as a biomarker of radiation-induced damage to human colorectal cancer cells. HCT116 cells were exposed to increasing doses of X-rays, and extracellular miRNAs were analysed by microarray. The results were correlated with the frequency of micronuclei. A total of 59 miRNAs with a positive correlation and 4 with a negative correlation between dose (up to 6 Gy) and extracellular miRNA expression were identified. In addition, for doses between 0 and 10 Gy, 12 miRNAs among those 59 miRNAs with a positive correlation were identified; for these extracellular miRNAs, a significantly positive correlation was observed between their expression and the frequency of micronuclei for doses up to 10 Gy. These results suggest that specific miRNAs may be considered as cell damage markers and may serve as secreted radiotherapy response biomarkers for colorectal cancer; however, the results must be further validated in serum samples collected from patients undergoing radiotherapy.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer and the fourth leading cause of cancer-related mortality

worldwide (1). CRC may be associated with pain and compromised quality of life (2,3). Although this type of cancer is mainly treated by surgery, radiotherapy is often used in the neoadjuvant setting to shrink the size of large tumours prior to surgery (4). When surgery is not an option, radiotherapy can be used in combination with chemotherapy as a radical treatment approach (5).

External beam radiotherapy has been used for >120 years (6) and is currently considered as a high-precision cancer treatment type that relies on inverse treatment planning and a magnitude of instrumental solutions that allow highly conformal dose delivery (7-10). Despite high dose conformity and intensity, some tumours exhibit a high degree of intrinsic radioresistance, leading to poor therapy outcome (11). Factors responsible for a high intrinsic radioresistance include tumour hypoxia and poor reoxygenation during treatment, repopulation between radiotherapy fractions, efficient repair of DNA damage and a high degree of heterochromatin (11-13).

With respect to CRC, there is considerable interest in devising biomarkers to predict pathological complete response (pCR) of the tumour to a combination of chemoradiotherapy (CRT) and surgery (14). Responsiveness of the tumour to preoperative CRT is crucial, as it may predict clinical outcome and even help decide whether post-CRT surgery is advisable. Han *et al* (14) analysed several clinical factors to determine their suitability as pCR biomarkers, and found that tumour location and post-CRT levels of the carcinoembryonic antigen (CEA) were promising predictive factors. However, the specificity and sensitivity of these as biomarkers were fairly low (area under the receiver operating characteristic curve comparing sensitivity vs. specificity = 0.638); therefore, additional biomarkers are needed.

Cell response to radiation can be estimated by analysing the release of microRNAs (miRNAs/miRs) to the surrounding medium. miRNAs are small, single-stranded non-coding RNAs that serve an important role in the regulation of gene expression. miRNAs bind to the 3'-untranslated regions of

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target mRNAs and control protein synthesis by increasing mRNA degradation or inhibiting translation (15). miRNAs released extracellularly are contained in extracellular vesicles and circulate in the body (16). miRNA levels and serum composition are promising biomarkers of cancer detection and progression in various types of cancer, including CRC (17-20).

Selected miRNA levels in the serum of patients who underwent radiotherapy for head and neck cancer were found to be correlated with disease remission (21,22). Promising results have also been reported for other types of cancer, including breast or head and neck cancer (23,24).

We became interested in identifying miRNAs that could be used to predict the response of colorectal tumours to radiotherapy. Since miRNAs isolated from the serum of patients undergoing treatment reflect the response of both cancer and normal cells to radiation, it was decided to start the analyses with an *in vitro* study using a CRC cell line. This approach aims to identify cancer cell-specific changes in the extracellular miRNA content, which, at a later step, can be validated in patient serum. In the present study, the expression of extracellular miRNAs secreted from irradiated HCT116 CRC cells was quantified by microarray and correlated with the frequency of cytogenetic damage. Cells were exposed to single-fraction X-irradiation to assess the basal response of the cell; however, fractionated irradiation is performed in clinical radiotherapy (e.g., 2 Gy/day x 25 fractions). Delivering a small fraction of the total radiation dose allows time for normal cells to repair themselves between treatments, thereby reducing side effects.

Materials and methods

Cell preparation and culture. The human colorectal cell line HCT116 was purchased from RIKEN BioResource Center. HCT116 cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated foetal bovine serum (Japan Bioserum, Co., Ltd.) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37°C and 5% CO₂.

Irradiation. X-ray irradiation (150 kVp, 20 mA with 0.5-mm aluminium and 0.3-mm copper filters) was performed using an X-ray generator (MBR-1520R-3; Hitachi Medical Co., Ltd.), with a distance of 45 cm between the focus and target. The dose was monitored with a thimble ionisation chamber placed next to the sample during irradiation. The dose rate was 1 Gy/min.

Apoptosis assay. The apoptotic cells were examined via direct immunofluorescence flow cytometry (Cell Lab Quanta™ Sc MPL; Beckman Coulter Immunotech). Cells harvested using a single pipette were washed twice using Annexin V Binding Buffer (cat. no. 422201) and immunostained following the manufacturer's instructions (BioLegend, Inc.). Following addition of fluorochrome-labelled protein, Annexin V-FITC (cat. no. 640906; BioLegend, Inc.), and propidium iodide (PI; MilliporeSigma), fluorescence intensity was quantified using flow cytometry (Cell Lab Quanta™ Sc MPL) and Kaluza Analysis Software (ver.2.0; Beckman Coulter, Inc.).

Cytokinesis-block micronucleus (CBMN) assay. The CBMN assay was conducted based of the criteria described by Fenech (25). After exposure of cells to X-irradiation, 15 µg/ml cytochalasin B (Cyt-B; FujiFilm Wako Pure Chemical Corporation) was added to RPMI-1640 medium, and then the cells were harvested after 3 days, which is when the maximum population of binucleated cells (BNCs) could be observed. Harvesting was performed by fixing the cells with 99% methanol at -20°C for 3 min and staining with 1 µg/ml Hoechst 33342 (MilliporeSigma) for 30 min at room temperature. The evaluation was conducted using a fluorescence/bright-field microscope at x400 magnification (IX71; Olympus Corporation). The BNC fraction was scored per slide for at least 10³ BNCs. At the same time, the nuclear division index (NDI) was determined from the frequency of >500 viable cells ('N' indicates the number of viable cells counted per slide) with 1, 2, 3 or 4 nuclei (M1, M2, M3 or M4, respectively) and calculated using the formula, NDI = [M1 + (2 x M2) + (3 x M3) + (4 x M4)]/N by fluorescence microscopic observation (25).

RNA extraction. Total extracellular RNAs released from irradiated cells (four replicates performed in parallel) were extracted from the cell culture supernatant using ISOGEN II (Nippon Gene Co., Ltd.), according to the manufacturer's instructions. The collection of RNAs in the cell culture supernatant was performed on day 3 after cell irradiation. This time point was decided as maximum RNA concentration in the cell culture supernatant and maximum percentage of apoptotic cells (26). The concentration of extracted RNAs was examined using Quant-iT RiboGreen RNA Reagent and Kit (Thermo Fisher Scientific, Inc.) and Fluoroskan Ascent (Thermo Fisher Scientific, Inc.). In addition, the peaks of small RNAs were confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The purity and concentration of extracellular RNAs were assessed using the NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The RNA samples had 260/280 nm absorbance ratios of 1.8-2.0.

Microarray analysis. Cy3-labelled miRNA was synthesised from 100 ng total RNA of X-irradiated and non-irradiated cell culture supernatant using miRNA Complete Labelling Reagent and Hyb kit (Agilent Technologies, Inc.). A SurePrint G3 human miRNA microarray slide (8x60 K, Ver. 21.0) was hybridised with the Cy3-labelled miRNA in a hybridisation solution prepared with a Gene Expression Hybridisation kit (Agilent Technologies, Inc.), following the manufacturer's instructions. Cy3 fluorescence signal images on the slide were obtained by using a microarray scanner (Agilent SureScan Microarray Scanner G 2600 D; Agilent Technologies, Inc.) and processed using the Feature Extraction version 10.7 software (Agilent Technologies, Inc.) based on the manufacturer's instructions. In the present study, miRNA samples were collected from cell culture supernatants and the extracellular miRNA content was examined. The expression data were processed using GeneSpring GX14.5 software (Agilent Technologies, Inc.) for normalisation to percentile shift 90% of all values on the respective microarrays, followed by normalisation of the median expression level of all samples, since using an internal control, such as U6, is not appropriate.

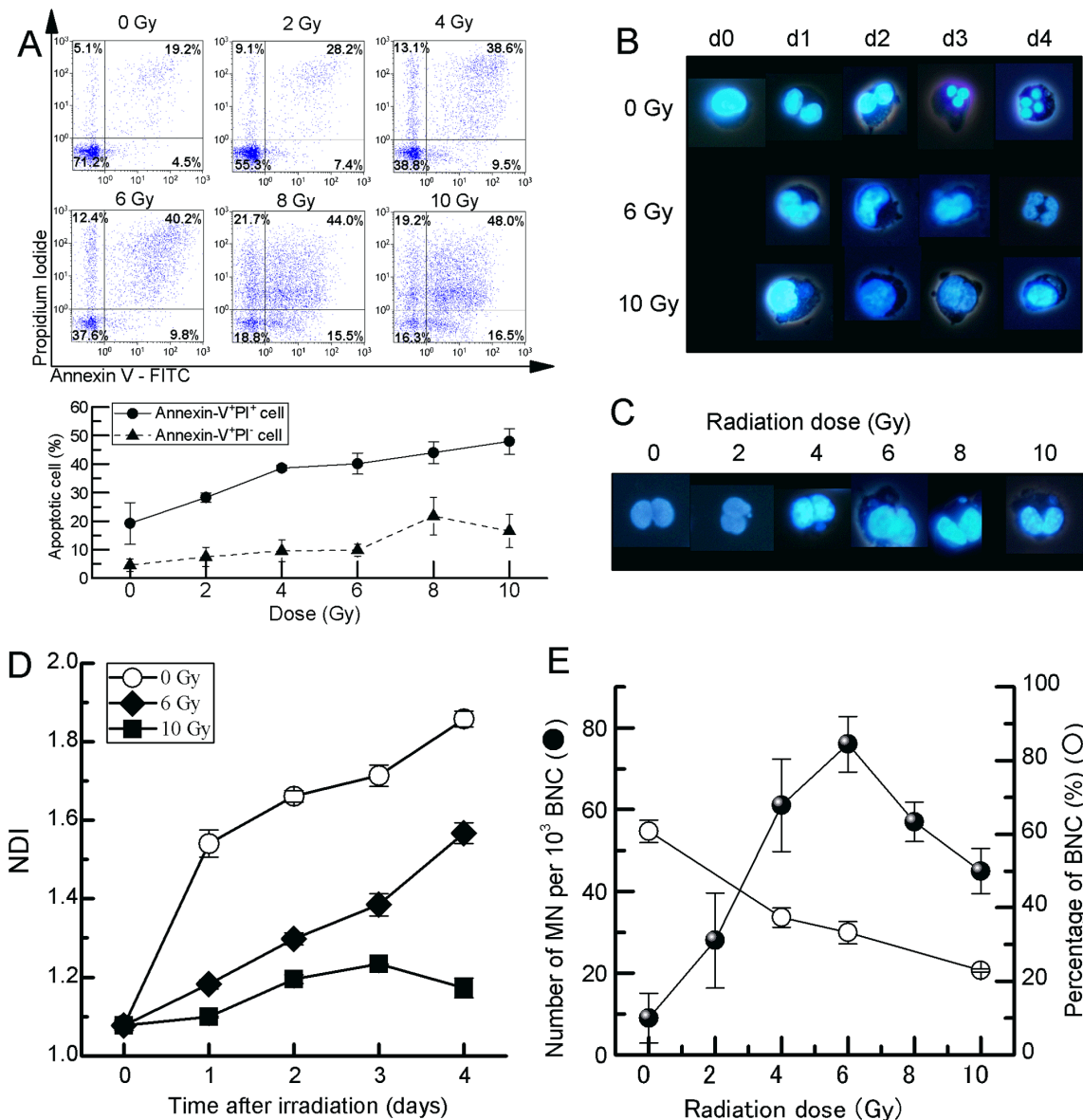


Figure 1. Analysis of CBMN assay in HCT116 cells exposed to X-irradiation. (A) Representative flow cytometry images and percentage of apoptotic cells, (B) representative images of nuclear division after irradiation, (C) BNCs with MN, (D) response of NDI and (E) MN frequency. The cell behaviour in HCT116 cells exposed to 2-10 Gy X-irradiation was observed until day 4. Apoptotic analysis and MN frequency in cells exposed to 2-10 Gy X-irradiation was estimated using flow cytometry (Annexin V⁺/PI⁺ early apoptosis and Annexin V⁺/PI⁺ late apoptosis) and the CBMN assay, respectively. The cells were cultured with Cyt-B, and MN and NDI were scored at 72 h after exposure to X-irradiation. The values are the mean \pm SE of four separate experiments. CBMN, cytokinesis-block micronucleus; MN, micronuclei; NDI, nuclear division index; BNC, binucleated cell; Cyt-B, cytochalasin B.

The lower raw data signals (lower cut-off of 50) were removed. In addition, the coefficient of variation was also set to <50.0% to remove miRNAs with large variations between experiments in advance. The average expression of each miRNA from cells exposed to X-irradiation at 2-10 Gy was compared to control within this normalised dataset (Fig. S1). The results were visualised with the help of heat maps. The microarray low data in the present study were uploaded onto the Gene Expression Omnibus database (GSE184174; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184174>).

Statistical analysis. Statistical analysis was performed using OriginLab software version 9.1 (OriginLab) and Excel 2013 (Microsoft Corporation) with the add-in software Statcel 3 (OMS Publishing, Inc.). The significant differences in miRNA

expression analysis and cell damage analysis were determined by Pearson's correlation test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of HCT116 cell damage from X-irradiation. We previously reported that the clonogenic potential of HCT116 cells exposed to high-dose-rate X-irradiation (1 Gy/min) is reduced in a dose-dependent manner, and the number of apoptotic cells also increases in a dose-dependent manner (26). The percentage of late apoptotic cells (Annexin V⁺/PI⁺) and early apoptotic cells (Annexin V⁺/PI⁻) by exposure to 2-10 Gy X-irradiation was found to be 19-50% and 5-17%, respectively, and it increased in a dose-dependent manner (Fig. 1A). Based

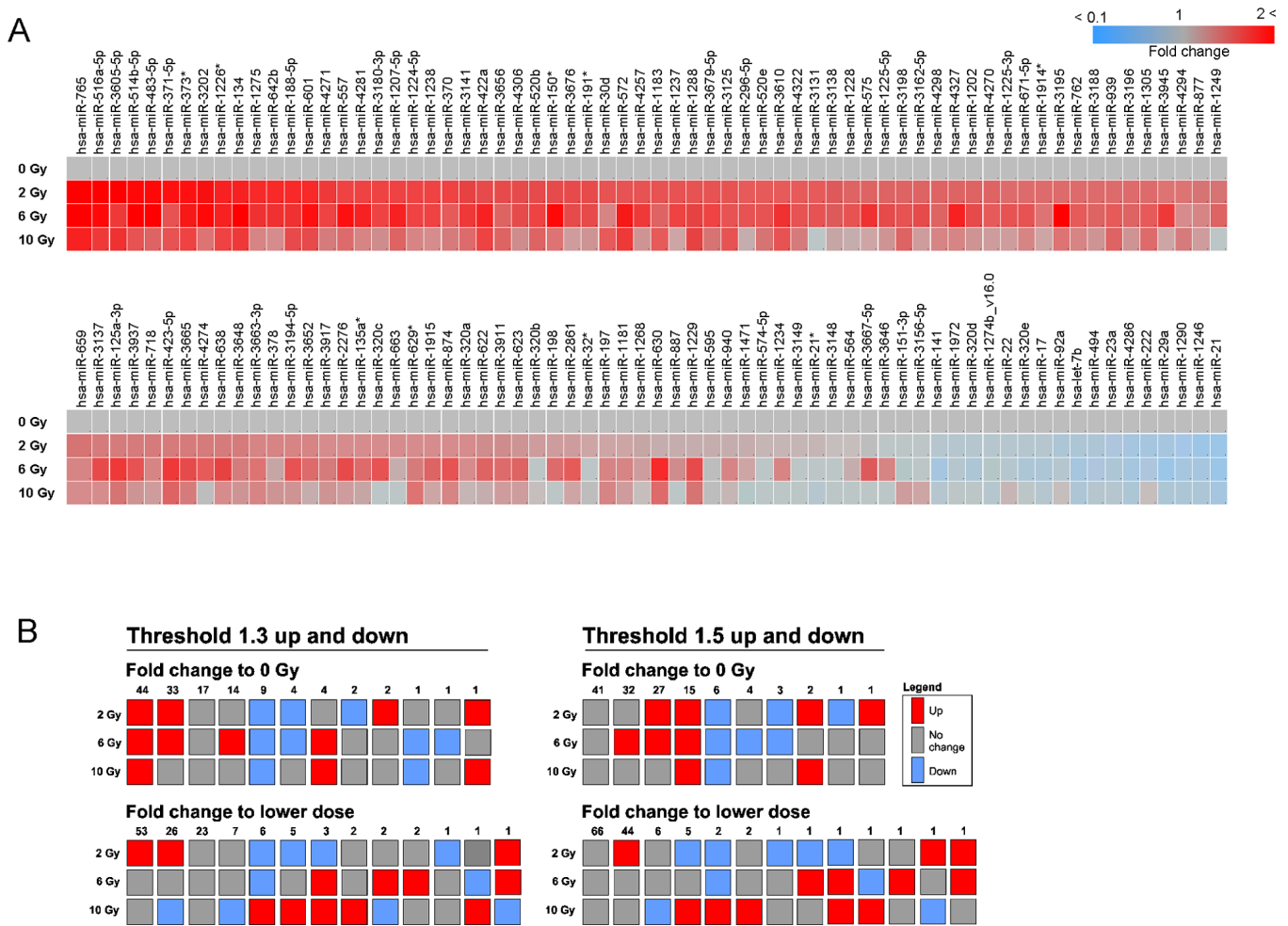


Figure 2. Dataset of miRNAs in focus. (A) Expression of extracellular miRNAs is displayed using a heat map. (B) miRNAs that were up- or downregulated 1.3- or 1.5-fold compared with 0 Gy (left) or the neighbouring lower dose (right) are shown as colour tiles. Upregulation, downregulation and no change are shown as red, blue or grey, respectively. miRNA, microRNA.

on these responses, a CBMN assay was performed using fluorescence microscopy to elucidate whether these cells exhibit dose-dependent nuclear damage by ionising radiation (Fig. 1B and C). The NDI, which is a marker of cell proliferation in cultures and is considered as a measure of general cytotoxicity (Fig. 1B), was significantly decreased in HCT116 cells between days 1 and 4 after exposure to 6 and 10 Gy compared with non-irradiated controls (Fig. 1D). As regards the frequency of micronuclei (MN), an increase (76.0 ± 6.81 MN/103 BNCs) was observed for doses up to 6 Gy; however, a decreased frequency was observed at doses >6 Gy (Fig. 1E).

Extracellular miRNA expression following exposure to X-irradiation. To determine the miRNAs released from HCT116 cells X-irradiated with 2-10 Gy, the cell culture supernatants were collected, and miRNAs were isolated at day 3 when the maximum ratio of apoptotic cells and MN frequency were reached (26,27). These samples were applied to 8x60 K format miRNA microarrays, and 132 human miRbase-annotated miRNAs were detected (Table SI, Fig. 2A). To identify miRNAs responding in a radiation dose-dependent manner, the miRNAs up- and downregulated by 1.3- or 1.5-fold were considered, as the number of detectable miRNAs was too

small (1 or 0) at an up-/downregulation threshold of 2-fold. Following exposure to 2-10 Gy, 44 and 15 miRNAs were upregulated by 1.3- and 1.5-fold, respectively, vs. the 0 Gy control group. In addition, 9 and 6 miRNAs were downregulated vs. the 0 Gy control group. On the other hand, no miRNAs were stepwise up- or downregulated by 1.3- and 1.5-fold, respectively, compared with the adjacent lower dose in any of the combinations (2 vs. 0, 6 vs. 2 or 10 vs. 6 Gy). There were still some miRNAs responding in accordance with the 6-Gy peak seen for the MN frequency. When comparing to the adjacent lower dose, patterns of up-up-down/up-up-no change and down-down-up/down-down-no change were found for 1 (miR-3195: 1.3- and 1.5-fold upregulation), 6 (miR-17, miR-29a, miR-98, miR-320d, miR-320e and miR-4286: 1.3-fold downregulation) and 2 (miR-29a and miR-98: 1.5-fold downregulation) miRNAs, respectively. The absolute level of miRNAs may also be a relevant factor allowing for detection in the serum, and miR-572, miR-939 and miR-3610 were the most highly expressed miRNAs on average (Table SI).

To elucidate the statistical correlation between radiation doses and miRNA expression, the 132 miRNA dataset was used, which was created based on the criteria described in Materials and methods. Using samples between 0 and 6 Gy, 59 miRNAs

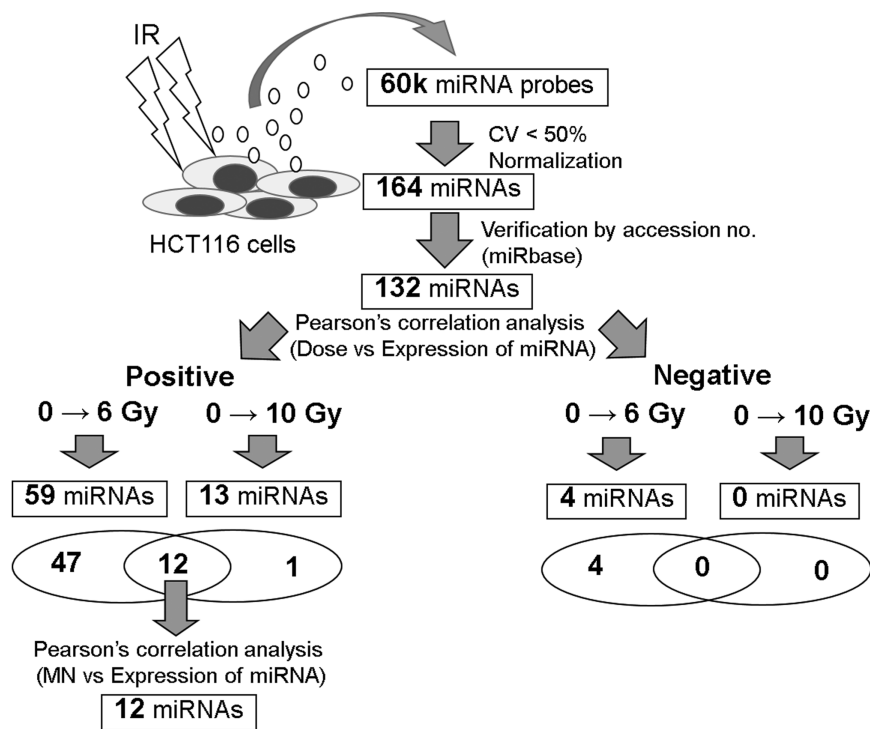


Figure 3. Schematic illustration of miRNA analysis. From the miRNA microarray data, initial filtering is displayed. Pearson's correlation analysis was performed between radiation dose and miRNA expression on the 132 filtered miRNAs of focus for this study. The 12 miRNAs exhibiting a significant positive/negative correlation between MN frequency and miRNA expression were analysed for both the 0-6 Gy and 0-10 Gy ranges. IR, ionizing radiation; miRNA/miR, microRNA; MN, micronuclei.

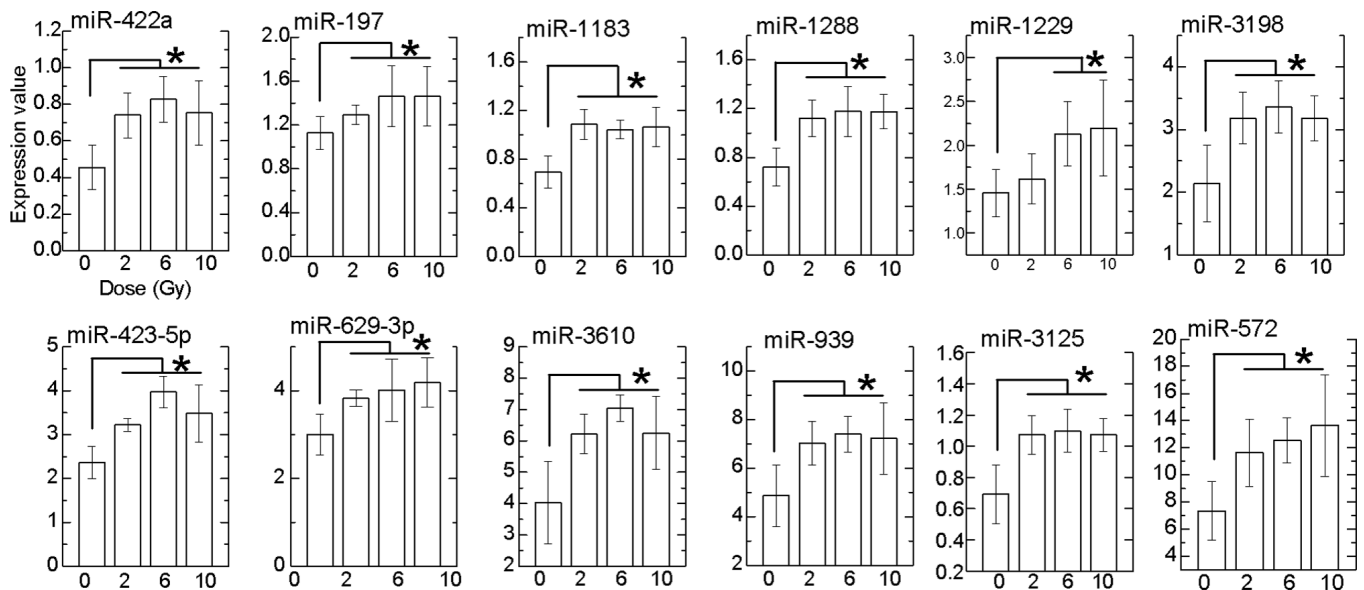


Figure 4. Relationship between radiation dose and miRNA expression. The 16 experimental samples exposed to X-irradiation were analysed, and bar charts of 12 miRNAs in each dose (0, 2, 6 and 10 Gy) are shown. * $P < 0.05$ by Tukey's test. miRNA/miR, microRNA.

exhibiting a positive correlation and 4 miRNAs exhibiting a negative correlation were identified (Fig. 3, Table SII). In addition, for samples between 0 and 10 Gy, 13 miRNAs exhibiting a positive correlation were identified. Of these, 12 miRNAs exhibited significantly upregulated expression at doses between 6 and 10 Gy compared with 0 Gy (Fig. 4). It is known that the maximum limiting dose of MN frequency in blood cells is 3-4 Gy (25). In the present study on HCT 116 cells, the maximum

limiting dose was up to 6 Gy (Fig. 1E). To predict the cellular damage and induction of apoptosis by the expression of extra-cellular miRNAs, we focused on the relationship between the 12 dose-dependently upregulated miRNAs and MN frequency (Fig. 5A), and the percentage of Annexin V⁺/PI⁺ cells (Fig. 5B). A significantly positive correlation of all miRNAs (miR-422a, $R=0.77$; miR-197, $R=0.50$; miR-1183, $R=0.63$; miR-1288, $R=0.73$; miR-1229, $R=0.84$; miR-3198, $R=0.74$; miR-423-5p, $R=0.78$;

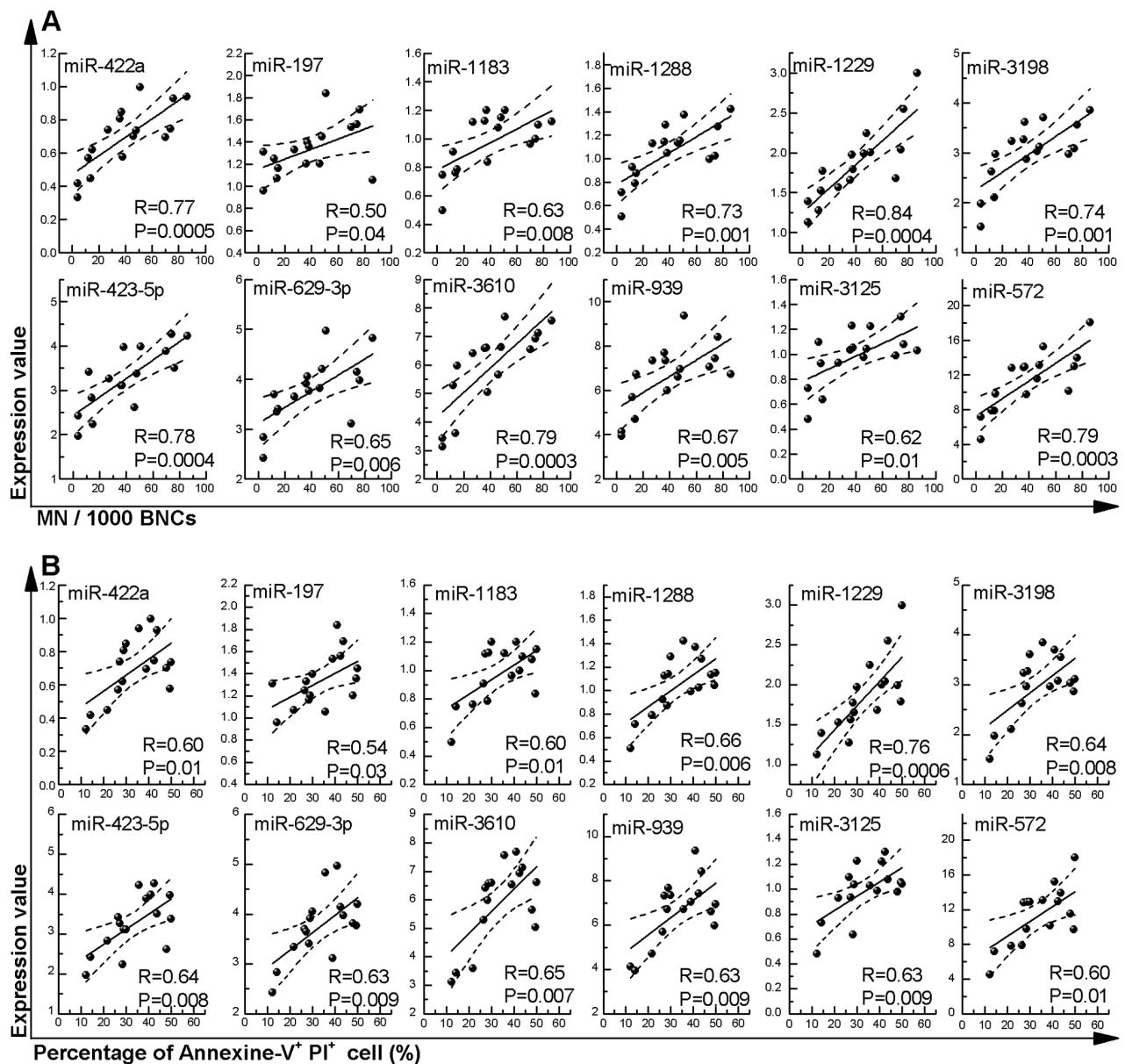


Figure 5. Relationship between (A) MN frequency and miRNA expression and between (B) percentage of Annexin V⁺/PI⁺ cells and miRNA expression. The 16 experimental samples exposed to 0-6 Gy X-irradiation were analysed, and cellular MN frequency was correlated with extracellular miRNA expression. The correlation line (black line), 95% upper confidence limit and lower confidence limit (dotted lines) are shown. R, Pearson's correlation coefficient. MN, micronuclei.

miR-629-3p, R=0.65; miR-3610, R=0.79; miR-939, R=0.67; miR-3125, R=0.62; and miR-572, R=0.79) was observed between the expression level and MN frequency (Fig. 5A). In addition, a significantly positive correlation of all miRNAs (miR-422a, R=0.60; miR-197, R=0.54; miR-1183, R=0.60; miR-1288, R=0.66; miR-1229, R=0.76; miR-3198, R=0.76; miR-423-5p, R=0.64; miR-629-3p, R=0.63; miR-3610, R=0.65; miR-939, R=0.63; miR-3125, R=0.63; and miR-572, R=0.60) was also observed with the percentage of Annexin V⁺/PI⁺ cells (Fig. 5B).

Discussion

The aim of the present study was to quantify the expression of extracellular miRNAs released from HCT116 CRC cells exposed to high-dose single-fraction ionising radiation to

identify predictive extracellular markers of radiotherapeutic CRC cell damage.

The dose-dependent radiation response at the level of cell nuclear damage, MN (Fig. 1), was in line with the previously reported reduced clonogenic potency for doses up to 6 Gy (26). This increased MN frequency indicates increased number of apoptotic cells. At doses >6 Gy, increased cell death or cell division arrest most likely contributed to the lower frequency of MN, since a larger proportion of cells with severe damage may have undergone apoptosis at the 72-h time point following exposure to X-irradiation.

In radiation biology, the CBMN assay is a radiation biodosimetry tool that uses peripheral blood lymphocytes and has an excellent dose dependency (28), contributing to dose estimation within the range of 2-6 Gy in cases of accidental radiation exposure (29). Clinically, 50 Gy/25 fractions or

25 Gy/5 fractions (30), which corresponds to 2.5 Gy/fraction, are delivered to the target region for CRC. Therefore, the exposure conditions of the present study are in the clinical range for a single fraction.

It is extremely difficult to analyse the target tissue cells located in deep regions using the CBMN assay in patients undergoing radiotherapy. However, it is possible to use body fluids to detect radiation response markers. A total of 60 specific miRNAs that were released extracellularly and their increased or decreased expression after exposure to X-radiation correlated with dose were identified (Fig. 4). The 12 selected miRNAs included those positively correlated to dose when analysing both 0-6 and 0-10 Gy, which shows that there was a lower expression and MN frequency at 10 Gy compared with those at 6 Gy. According to the American Society for Radiation Oncology and the Japanese Society for Radiation Oncology (31,32), the clinical fractionation scheme for CRC tissue is delivering ~50 Gy as a total dose in 25 fractions (2 Gy/day). Therefore, miRNA expression in body fluids, such as the serum, has a potential as a monitoring tool within this dose range, whether or not cancer cells are damaged.

In research on biomarkers from body fluids, the peripheral blood, including the metabolites from cancer tissue, has been evaluated for diagnosis and prognosis prediction. In CRC, CEA and carbohydrate antigen 19-9 antigens are considered as representative markers in cancer screening tests (33). Cancer researchers have recently become interested in detailed screening of proteins, DNA and RNA in body fluids for clinical applications to further improve the accuracy of cancer detection (34-37). In recent years, miRNAs have been reported to be highly promising as cancer biomarkers (38). miRNAs regulate various biological processes in cells, and their expression fluctuates with the DNA damage response to radiation (39). Thus, extracellular miRNAs in patients receiving radiotherapy may reflect a radiation-specific response and may be useful for the determination of radiation dose.

Within the data presented herein, it was observed that 8 of 12 miRNAs (miR-422a, miR-197, miR-1183, miR-1288, miR-1229, miR-423-5p, miR-939 and miR-572) have a function in CRC. Intracellular expression of miR-422a, miR-572 and miR-939 serve a role in tumour suppression; miR-422a targets AKT1 and MAPK1; miR-572 targets the MOP-1 pathway activated by STAT3 and miR-939 targets NF- κ B (40-44). Of those, miR-572 and miR-939 were also among the three most highly expressed miRNAs, with the highest chance of detection in the blood. miR-197, miR-1183 and miR-1288 are associated with CRC response (upregulation) to chemotherapy and/or radiotherapy (45-47). miR-423-5p and miR-1229 were reported as diagnostic circulating biomarkers of CRC (48,49). However, the remaining miRNAs (miR-3198, miR-629-3p, miR-3610 and miR-3125) have no previous reports in the field of CRC. Therefore, the phenomenon seen in this study is that the dose-dependent intracellular response to the ionising reaction affected the mRNA release system, including extracellular vesicles. Therefore, these 12 miRNAs may serve as potential biomarkers of radiation response in CRC cells.

Radiotherapy causes activation of several processes in the tumour microenvironment, such as inflammation, cycling hypoxia, immunomodulation, revascularisation, and extracellular matrix remodelling coordinated by cancer-associated

fibroblasts and fibrosis (50). The release of miRNAs from tumour cells may contribute to these effects, and it is possible that metabolite control differs among patients with cancer. Of note, there is still a need for improved estimates of which patients are responding well or less well to treatment; therefore, these miRNAs may serve as markers with the purpose of optimising target exposure to the radiation dose during radiotherapy. Further detailed *in vitro* and *in vivo* studies are needed to validate these data and to analyse if the expression pattern of these specific miRNAs also contributes to the radiosensitivity of CRC. A limitation of the present study was the lack of investigation of miRNA expression in clinical samples (e.g., colon cancer vs. normal adjacent non-cancerous samples). To verify the evidence on the 12 released extracellular miRNAs, the reproducibility in animal models and/or clinical specimens, and the response of CRC cell exposed to fractionated irradiation, should be further investigated in the future. In addition, we plan to test the role of these 12 miRNAs in endocytosis by CRC cells exposed to high-dose rate ionising radiation.

In conclusion, the findings of the present study suggest that specific extracellular miRNAs have the potential to serve as cell injury markers induced by single high doses of ionising radiation in CRC cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SM, TU, YMa and LL designed the study, prepared the manuscript draft and substantively participated in revising the manuscript. SM, TU, YMo and MC contributed by analysing the biological data. TU and AW contributed by normalizing by the microarray data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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