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MicroRNA-205 enhances the radiosensitivity of canine oral melanoma cells by inhibiting E2F1

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Abstract

Canine oral malignant melanoma (CoMM) is a highly aggressive tumor associated with a poor survival due to both local disease progression and high metastatic potential. Radiotherapy is often applied as the treatment for dogs with melanoma. However, the therapeutic effect of it may be limited due to the relative radio-resistance. Recent studies have demonstrated that microRNAs (miRNAs) play an important role in radiosensitivity. However, little is known with regard to the association of radiosensitivity with miRNAs in canine melanoma cells. In the present study, we showed that miR-205, which is downregulated in CoMM tissues compared to its expression in normal oral tissues, promoted the radiosensitivity of canine melanoma cells. In addition, our results indicated that the enhancement of radiosensitivity by miR-205 resulted, at least in part, from negative regulation of E2 transcription factor 1 (E2F1)-ATM signaling, which finding was validated by using the methods of knockdown of E2F1 and knockout of ATM for CoMM cell lines. However, other mechanisms regulated by miR-205 were considered to contribute to radiosensitivity in cutaneous melanoma cells, because downregulation of E2F1 did not decrease the expression level of ATM. Taken together, these findings suggest that miR-205 plays a role in radiosensitivity of CoMM cells by inhibiting E2F1 and indicate that utilization of this miRNA as a radiosensitizing factor might be a new strategy for treatment of CoMM.

Key Words: ATM, E2F1, melanoma, miR-205, radiation

Introduction

Canine oral malignant melanoma (CoMM) is a common malignancy of dogs and has a poor prognosis. CoMM is locally invasive and metastasizes readily, as in the case of human melanoma[16,24)]. It is considered that canine melanoma is an important preclinical model of

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the human one, because of the similarity of the expression profile of several genes and clinical behavior between both melanomas[9]. In humans, skin melanomas are locally treated with surgical resection following radiation therapy[4]. Also, surgery and radiation therapy as local control modalities are recommended for CoMM. The median survival time of dogs with surgically treated CoMM are approximately 21 to 30, 6 to 27, and 5 to7 months for animals with stage I, II, and III disease, respectively[15,28]. On the other hand, that for treatment with radiation therapy is 25.3, 9.3, 5.4, and 2.7 months with stage I, II, III, and IV, respectively[12]. Thus, it seems that radiotherapy has an effect almost equivalent to that of surgery for CoMM. Moreover, because of the recent increasing veterinary use of radiation therapy equipment, CoMM animals have a greater opportunity to receive radiotherapy. However, successful radiotherapy depends on tumor radiosensitivity; and CoMM recurrence after radiotherapy often occurs. Therefore, it is necessary to clarify the underlying molecular mechanisms of radioresistance of CoMM to improve the outcome of CoMM animals treated with radiotherapy.

MicroRNAs (miRNAs) are small-non-coding RNAs that regulate gene expression at the post-transcriptional level through complete or incomplete complementary binding to the 3'-untranslated region (3'-UTR) of their target genes[1]. Recent studies have shown that miRNAs affect the therapeutic response to radiotherapy, which finding promises to lead to identification of potential therapeutic targets to improve tumor radiosensitivity[7,25,30].

Previous studies have demonstrated that miR-205 expression is markedly down-regulated in canine and human melanoma cells and acts as a tumor suppressor by negatively regulating *E2 transcription factor 1* (*E2F1*), *zinc-finger E-box binding homeobox 2* (*ZEB2*), and *erb-b2 receptor tyrosine kinase 3* (*ERBB3*) in human melanoma[14,19,20]. Furthermore, miR-205 has been demonstrated to be related to cellular radioresistance. In human nasopharyngeal carcinoma, miR-205 inhibits radiosensitivity by directly targeting *Phosphatase And Tensin Homolog* (*PTEN*)[22]. On the other hand, miR-205 enhances radiosensitivity in breast cancer cells[29]. Thus, although miR-205 contributes to the cellular response to radiation, it might exhibit various functions, which depend on the types of cancer.

Ataxia telangiectasia mutated (ATM) serine/threonine kinase plays a critical role in the repair of DNA double-strand breaks, which can be induced by ionizing radiation and certain chemotherapeutic agents[5]; and it transduces the DNA damage repair signal to downstream proteins by phosphorylation[13]. It is also known that somatic mutations in *ATM* occur in many human tumor types[9]. Such mutations possibly sensitize cancer cells to radiotherapy or chemotherapy-induced DNA damage, although *ATM* mutations are known to be associated with worse prognosis in several cancers. Also, it has been found that ATM inhibition enhances the response to ionizing radiation[27]. On the other hand, a recent study suggests that *ATM* is a target gene of E2F1[2]. E2F1 transcription factor is post-translationally modified and stabilized in response to various kinds of DNA, resulting in regulation of the expression of cell cycle and pro-apoptotic genes. In addition, it has been reported that E2F1 directly contribute to DNA repair and genome maintenance in the DNA damage response[6].

In this study, we examined the involvement of miR-205 in the cellular sensitivity of canine melanoma cells to radiation. Here, we found that miR-205 plays a potential role as a radiosensitizing factor in the radiotherapy of canine melanoma.

Materials and methods

Cell culture: Canine melanoma cell lines, KMeC, CMM1, and CMeC-1 were a gift from The

University of Tokyo (Dr. Nakagawa)[11]. KMeC and CMM1 cells were derived from CoMM, and CMeC-1 cells were from a canine cutaneous melanoma. The cells were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Gibco, Waltham, MA, USA) at 37°C in a humidified atmosphere with 5% CO_2.

X-irradiation: Irradiation was performed using a Radio flex 350 (RF350; 0.5 mm Al filter and 0.3 mm Cu filter, 250 kVp, 14 mA, Rigaku, Tokyo, Japan) and the dose rate was 1.0 Gy/min.

Cell viability and clonogenic assay: To evaluate the cell viability, we seeded cells at 0.5×10^5/dish into 3 cm dishes the day before Iozining radiation (IR). At 72 hr after IR, the number of viable cells was determined by performing the trypan blue dye-exclusion test. For the clonogenic assay, 500 (CMeC-1 and KMeC), 1000 (CMM1) or 5000 (ATM-KO KMeC) cells were plated in 3-cm tissue culture dishes. After 24 hr, the cells were treated with a single dose of radiation (0, 1, 2 or 4 Gy). For the cells transfected with a plasmid vector or siRNA, the transfection was performed on the day after seeding and the cells were irradiated after 24 hr of transfection. The cells were incubated for 7–9 days after IR. Colonies were stained with crystal violet. Colonies containing $>$ 0 cells were counted under a microscope. The survival fraction was calculated as (the number of colonies / the number of cells plated)$_{\text{irradiated}}$ / (the number of colonies/the number of cells plated)$_{\text{non-irradiated}}$.

Antibodies: The following mouse monoclonal antibodies were used: anti-E2F1 monoclonal antibody (KH95; 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ATM monoclonal antibody (G-12; Santa Cruz). Horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Bio-rad (Osaka, Japan).

Cell transfection with plasmid vector or siRNA: To generate miR-205 or E2F1-overexpressed cell lines, we seeded into a 25-cm^2 flask at the concentration of 1.5×10^5 cells per flask the day before transfection. miRNASelect^{TM} pEP-hsa-mir-205 Expression Vector (pEP-miR-205; Cell Biolabs, Inc., San Diego, CA, USA) was used for stable expression of miR-205; and miRNASelect^{TM} pEP-miR Null Control Vector (pEP-null), as a negative control. For overexpression of E2F1, the cells were transfected with pcDNA3.1 harboring the canine E2F1 open reading frame (GenScript, Piscataway, NJ, USA) or pcDNA3.1 (as a negative control).

Short-interfering RNA (siRNA) for *E2F1* (5'-GACUCCUCGCAGAUCGUCAUCAUCU-3'; siR-*E2F1*; Invitrogen, Waltham, MA, USA) was used for transfection of the cells; and Stealth RNAi^{TM} siRNA negative control med GC (Thermo Fisher Scientific, Waltham, MA, USA) was used as a non-specific control siRNA.

The transfection with each plasmid vector or siR-*E2F1* was achieved by using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen) at a concentration of 1 μg of each plasmid vector/flask or dish or 1 or 10 nM siRNA, according to the manufacturer's Lipofection protocol. Stable selection was achieved by use of puromycin for pEP plasmid vectors and G418 for pcDNA3.1 plasmid vector.

Development of ATM knockout melanoma cell lines: ATM-knockout (KO) KMeC cells (2C10 and 3G9) were provided by Dr. Mizuno (manuscript in preparation). These cell lines were generated by using the CRSPR/Cas9 system targeting the canine *ATM1* gene.

Quantitative RT-PCR: Total RNA was isolated from cells by the phenol/guanidium thiocyanate method with DNase I treatment. For determination of mRNA expression levels, total RNA was reverse transcribed with a TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacture's procedures. To determine the expression of

miRNAs, we used TaqMan MicroRNA Assays (hsa-miR-205 and RNU6B; Thermo Fisher Scientific). Real-time PCR was then performed by using a THUNDERBIRD® Probe qPCR Mix (TOYOBO, Osaka, Japan). The relative expression level of miR-205 was calculated by the ΔΔCt method. *RNU6B* was used as an internal control.

Western blotting: Total protein was extracted from whole cells, and SDS-PAGE and electroblotting were performed according to the procedure described previously[20]. The antibodies were properly diluted with TBS-T containing 2% bovine serum albumin and 0.01% sodium azide. The loading control was prepared by re-incubating the same membrane with anti-β-actin antibody (1 : 5000, Sigma, St. Louis, MO, USA).

Statistical analysis: Each examination was performed in triplicate and independently repeated 3 times. All calculated data were compared by using the unpaired 2-tailed Student's *t*-test or One-way Anova following Tukey methods. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

MiR-205 acted as a tumor radiosensitizing factor in melanoma cells

To confirm whether miR-205 acts as a tumor radiosensitizing factor or promotes radioresistance in melanoma cells, we examined the cell viability and colony formation capacity of miR-205- or null-transfected canine melanoma cells (CMeC-1/205, CMeC-1/null, CMM/205, CMM/null, KMeC/205, and KMeC/null). The cells transfected with the miR-205 expression vector successfully exhibited a much higher expression level of miR-205 than the control cells (Fig. 1a). The cell growth was not significantly different between miR-205-overexpressed cells and control cells for all cell lines tested (Fig. 1b). Also, the cell-cycle distribution was not significantly different between these cells as judged by flow cytometric analysis using PI staining (data not shown). The post-IR viability of KMeC/205 cells was significantly lower than that of the KMeC/null ones (Fig. 2a). Also, the clonogenic ability of miR-205-overexpressed cells exposed to X-rays was significantly lower than that of the control cells for all cell lines (Fig. 2b and c). The transfection with the pEP plasmid vector did not affect the viability and the clonogenic ability of KMeC cells (data not shown).

MiR-205 enhanced radiosensitivity by targeting E2F1

We previously reported that miR-205 regulates E2F1 expression in and inhibits cell growth of human melanoma cells[19,20]. E2F1 protein has been shown to be important for DNA end resection and the formation of single-stranded DNA at double strand breaks (DSBs)[2]. As shown in Fig. 3, miR-205 overexpression resulted in decreased E2F1 expression in CMeC-1, CMM1, and KMeC cells. Therefore, to examine whether E2F1 could affect the radioresistance of melanoma cells, we prepared E2F1-overexpressed KMeC/null and KMeC/205 cells (null/null, null/E2F1, 205/null, and 205/E2F1; Fig. 4a). The cell viability of E2F1-overexpressed cells after IR was significantly higher than that of E2F1 non-overexpressed cells (Fig. 4b). The survival curves obtained from the colony formation assay using null/null, null/E2F1, 205/null, and 205/E2F1 cells exposed to X-ray are shown in Fig. 4c. In terms of the clonogenic ability, there was a significant difference between the 205/E2F1 cells and 205/null cells exposed to 1 Gy, but no difference between null/null and null/E2F1 at any dose. In contrast, we evaluated the effect of *E2F1* knockdown on the clonogenic ability of CMM1 cells. We used these cells, because they showed the highest expression level of E2F1 (Fig. 3). As shown in Fig. 4d, *E2F1* knockdown successfully decreased the clonogenic ability in a dose-dependent manner. Furthermore, consistent with

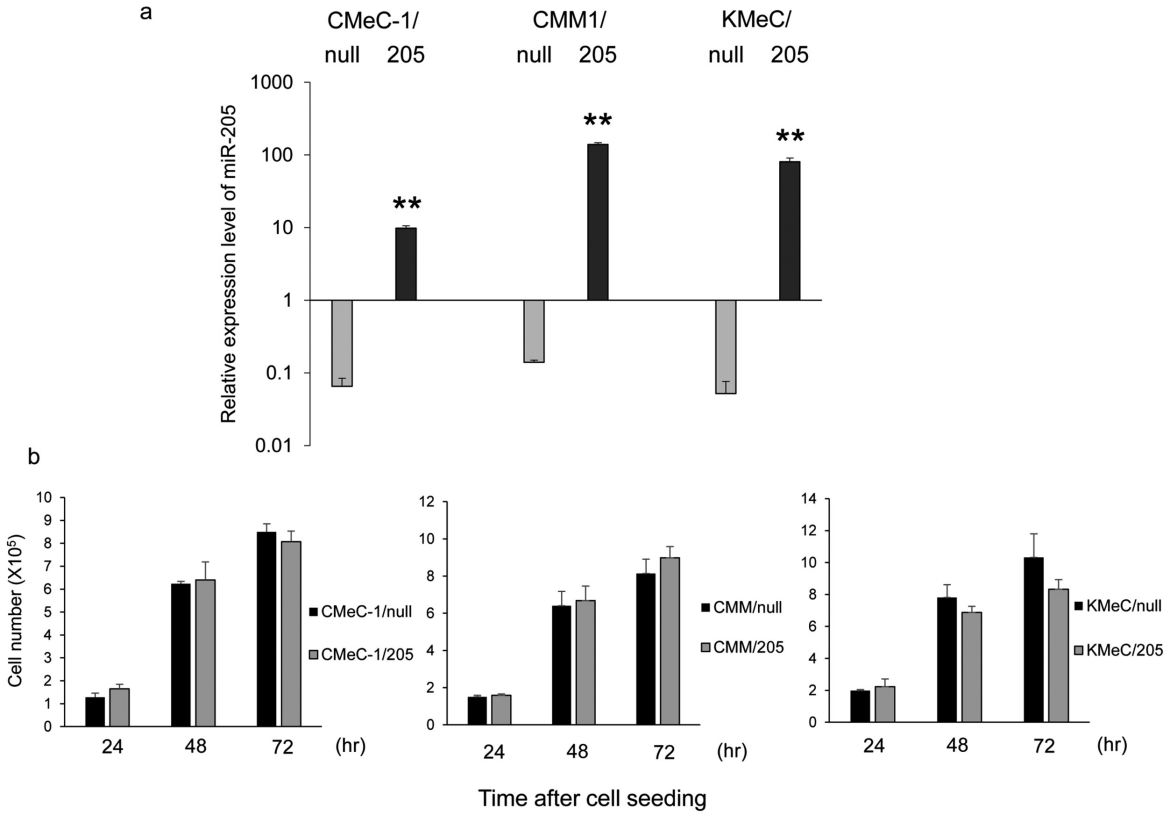

Fig. 1. Development of canine melanoma cell lines stably overexpressing miR-205. (a) Relative expression level of miR-205 in each cell line. The vertical axis was expressed by common logarithm. (b) The viable cell number was counted at 24, 48, and 72 hr after cell seeding. $^{**}P < 0.01$, for differences between miR-205-overexpressed cells and control cells. Data are expressed as the mean + SD (n = 3).

the findings in an earlier study[2], the silencing of *E2F1* decreased the expression level of ATM (Fig. 4d).

The expression level of ATM in miR-205-overexpressed CoMM cells was down-regulated and knockout of ATM enhanced radiosensitivity of KMeC cells

Figs. 3 and 4 indicated that miR-205 targeted *E2F1* and promoted radiosensitivity, but this mechanism remained unclear. It has been shown that E2F1 activates the ATM promoter and elevates both mRNA and protein levels of ATM[2]. ATM is a high molecular-weight protein serine/threonine kinase that plays an important role in the DSB-induced DNA damage response, in which ATM transduces a DSB damage/repair signal to downstream effector machinery by phosphorylating critical protein substrates[6]. Thus, we hypothesized that miR-205 inhibits the expression of E2F1 followed by a reduction in the ATM expression level. So, we examined the expression level of ATM in the cells indicated in Fig. 5a. As a result, the expression level of ATM was down-regulated by miR-205 overexpression in CMM1 and KMeC cells (Fig. 5a). On the other hand, CMeC-1/205 cells showed a higher level of ATM expression than CMeC-1/null. Next, we produced ATM-KO KMeC cells (clones, 2C10 and 3G9) to investigate how ATM affected the radiosensitivity of canine melanoma cells (Fig. 5b). As shown in Fig. 5c and d, we confirmed that the clonogenic ability of 2C10 and 3G9 cells exposed to 4 Gy was significantly lower than that of the wild-type KMeC (WT) cells.

To validate whether miR-205 enhanced the radiosensitivity of CoMM cells solely by modulating the E2F1/ATM signaling pathway,

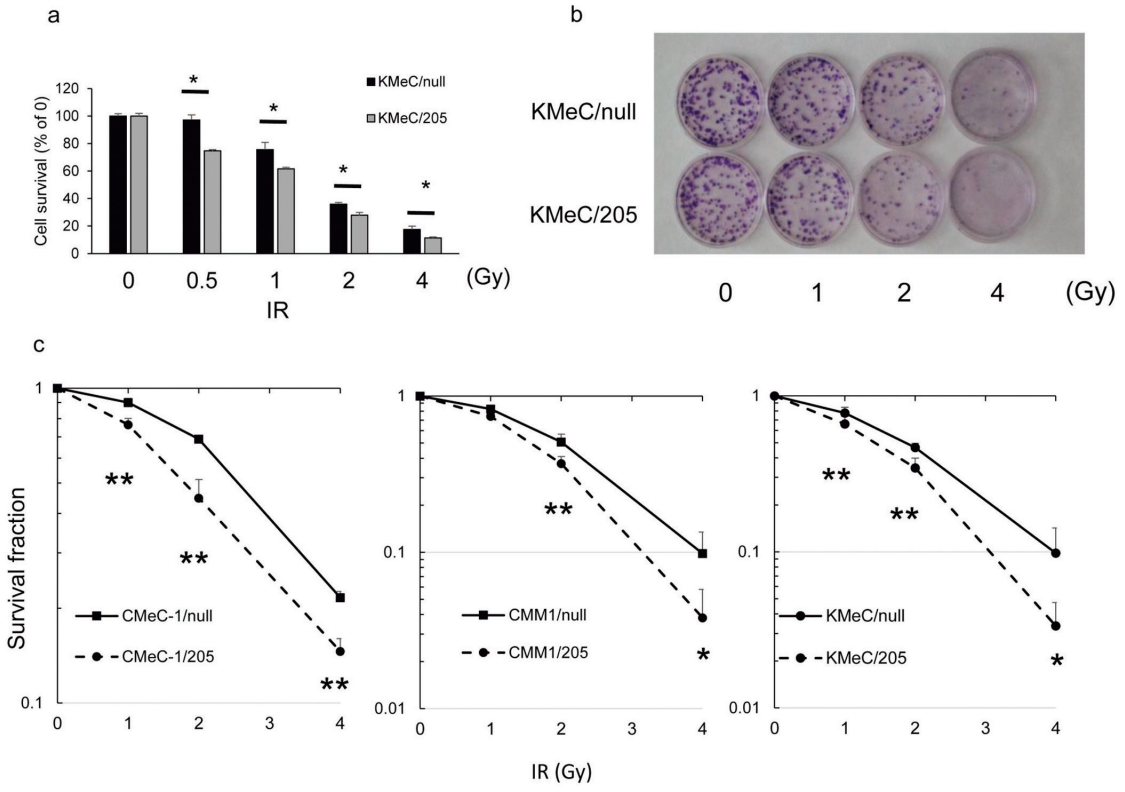

Fig. 2. miR-205 increased radiosensitivity of canine melanoma cell lines. (a) Number of viable KMeC/null and KMeC/205 cells at 72 hr after IR (0, 0.5, 1, 2, and 4 Gy). (b) Results of clonogenic assay using KMeC/null and KMeC/205 cells. (c) Dose-response curves of X-irradiated CMeC-1/null and CMeC-1/205 (left), CMM1/null and CMM1/205 (middle), KMeC/null and KMeC/205 cells[26] by clonogenic assay. $*P < 0.05$, $**P < 0.01$, for differences between the miR-205-overexpressed cells and the control cells. Data are expressed as the mean + SD (n = 3).

we performed the colony formation assay using ATM-KO KMeC cells transfected with pEP-null or pEP-miR-205. Each pEP plasmid vector was used for transient transfection, because the ATM-KO cells were already resistant to puromycin. As a result, the overexpression of miR-205 significantly increased the radiosensitivity of the ATM-KO cells (Fig. 5e).

Discussion

Radiation therapy fails to control a local lesion in some CoMM animals. So far, there are no reports with regard to biomarkers involved in the radiosensitivity of CoMM, although such biomarkers are obviously needed to improve the outcome of dogs with CoMM. A recent study reported that the expression level of miR-205 in CoMM tissues is significantly decreased compared with that in canine oral normal tissues[20]. In addition, other reports showed that miR-205 promotes radioresistance in nasopharyngeal carcinoma and esophageal squamous cell carcinoma or radiosensitivity in breast cancer cells[21,22,29]. Based on these findings, we hypothesized that miR-205 has some kind of effect on the radiosensitivity of canine melanoma cells and that miR-205 or miR-205 target genes could be utilized as a biomarker. In this study, we eventually confirmed that miR-205 acted as a tumor radiosensitizing factor in CoMM cells.

Recently, we suggested that miR-205 inhibits the growth of human melanoma cells by targeting *E2F1*[20], although no significant difference was observed between null and 205 cells in this study (Fig. 1b). Probably, the signaling pathways modulated by miR-205 are complemented by

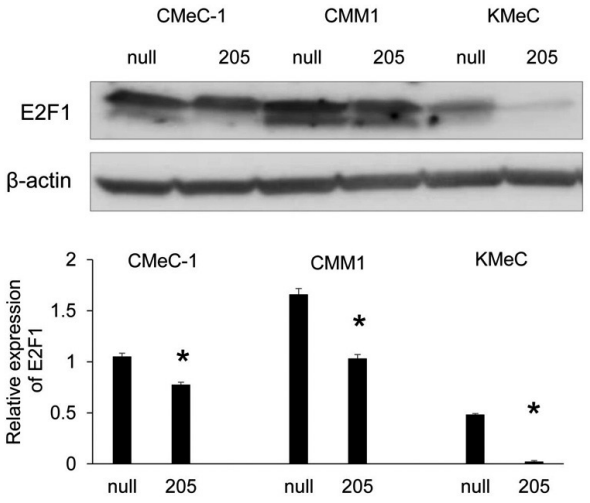

Fig. 3. Expression of E2F1 protein in CMeC-1, CMM1, and KMeC cells transfected with an miR-205 expression or a null vector as determined by Western blotting analysis (upper panel). The intensities of the recognized bands were analyzed by densitometry. The intensity of each band of E2F1 was normalized to that of β-actin (lower graph). $*P < 0.05$, for differences between miR-205-overexpressed cells and the control cells. Data are expressed as the mean + SD (n = 3).

other pathways through establishment of miR-205 overexpressed cells so that the cells can survive. Meanwhile, another ealier study demonstrated that E2F1 can stimulate the DNA repair signaling pathway and promote cell survival in response to DNA damage[3]. However, the relationship between this miR-205-E2F1 pathway and irradiation has remained unclear. Therefore, we investigated the radiosensitivity among cells with or without overexpression of miR-205 and/or E2F1. As was shown in Fig. 4c, the radiosensitivity of 205/null cells was significantly lower than that of 205/E2F1 cells. Furthermore, the silencing of *E2F1* increased radiosensitivity (Fig. 4d). These results indicated that miR-205 enhanced the radiosensitivity by targeting *E2F1*. However, there was no difference between null/null and null/E2F1 cells. The signaling pathway associated with E2F1 is probably up-regulated in CoMM cells due to down-regulation of miR-205, which negatively regulates the pathway. Therefore, the effects of E2F1 signaling pathway may be considered to reach a plateau in the cells without overexpressed miR-205.

ATM serine/threonine kinase plays a critical role in the cellular responses to IR-caused DNA damage[5] and transduces the DNA damage signal to downstream proteins[13]. A recent study suggests that *ATM* is a target gene of E2F1[2]. In this study, we validated that the expression level of ATM in miR-205-overexpressed CMM1 and KMeC cells was lower than that in the control cells and that the radiosensitivity of ATM-KO KMeC cells was significantly increased compared with that of the wild-type KMeC cells. Furthermore, silencing of E2F1 down-regulated the expression level of ATM and significantly decreased the clonogenic ability of CMM1 cells after IR. These results indicate that miR-205 contributed to the enhancement of radiosensitivity of CoMM cells through E2F1 down-regulation and subsequent ATM down-regulation. However, in contrast, the protein expression level of ATM was increased in the CMeC-1 cells that overexpressed miR-205. The mechanisms of miR-205 promotion of radiosensitivity might depend on the cell types or the kind of the original tissue, because CMM1 and KMeC cells were derived from CoMM and CMeC-1 cells were from a canine cutaneous melanoma. On the other hand, overexpression of miR-205 significantly increased the radiosensitivity of ATM-KO cells. This finding suggests that miR-205 affected other signaling cascades to regulate the radiosensitivity of melanoma cells, as well as E2F1-ATM pathway.

It has been reported that miR-205 inhibits radioresistance by targeting *ZEB1* and *Ubc13* in breast cancer cell lines[29]. So we examined whether miR-205 would affect the protein expression level of Ubc13 in canine melanoma cell line. However, no difference was shown between miR-205-overexpressed cells and the control cells (data not shown).

To further examine the mechanism of radiosensitivity involved in the function of miR-205, we established radio-resistant KMeC cells (KMeC-R, Fig. S1a) and evaluated the expression level of miR-205 between KMeC and

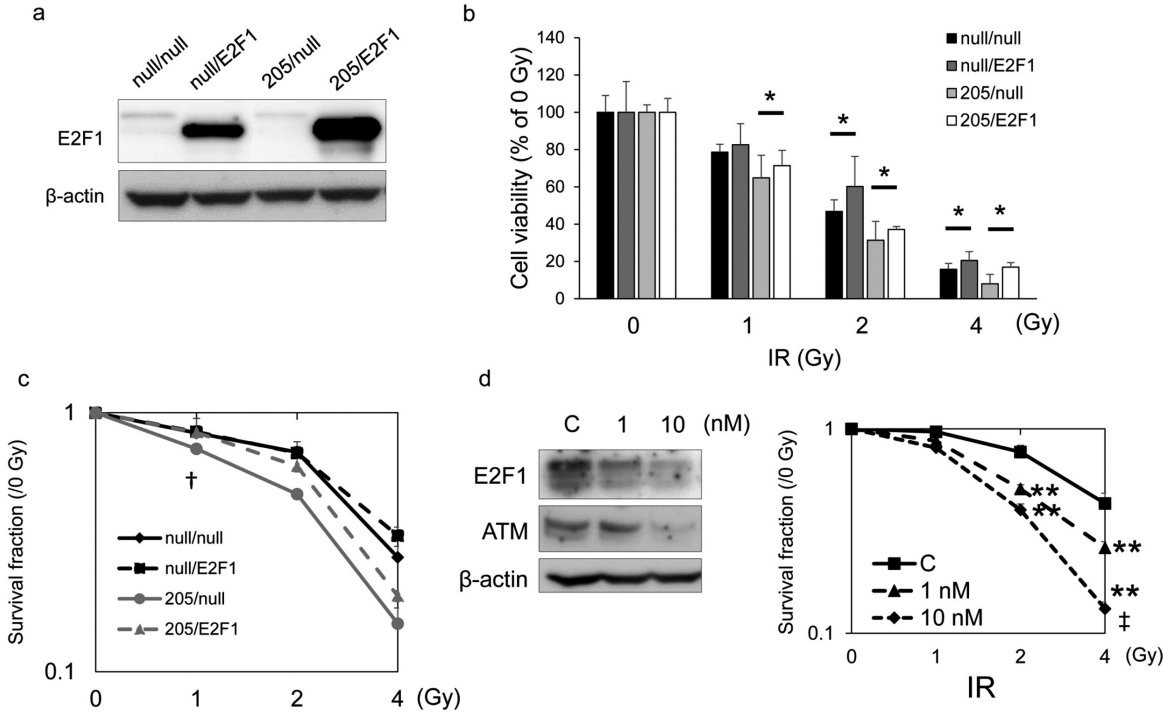

Fig. 4. Effect of E2F1 overexpression or knockdown on radiosensitivity of CoMM cells. (a) Protein expression level of E2F1 in null//null, null/E2F1, 205/null, and 205/E2F1 cells as determined by Western blotting analysis. (b) Number of viable cells at 72 hr after IR (0, 1, 2, and 4 Gy) of these cells. $*P < 0.05$, for differences between E2F1-transfected cells and null transfected ones. (c) Survival fraction of null//null, null/E2F1, 205/null, and 205/E2F1 cells after IR at the indicated doses. $\dagger P < 0.05$, for differences between 205/null cells and 205/E2F1 ones. (d) Protein expression levels of E2F1 and ATM (left panel) and survival curves of clonogenic assay in CMM1 cells transfected with control (C) or siR-*E2F1* at the indicated doses. $**P < 0.01$, for difference between siR-*E2F1* transfected cells and control siRNA-transfected ones. $\ddagger P < 0.01$, for difference between the cells transfected with siR-*E2F1* at 1 nM and 10 nM. Data are expressed as the mean + SD (n = 3).

KMeC-R. As a result, unfortunately, there was no difference in the expression level of miR-205 (Fig. S1b). This fact suggests that miR-205 is irrelevant to acquired radioresistance in CoMM cells. Additionally, we examined the relevance of the expression level of miR-205 to the response to radiotherapy in CoMM clinical samples to validate whether its expression level could predict the radiosensitivity. As shown in Fig. S2, the expression level of miR-205 tended to be related to a positive response to radiation therapy, but not significantly so. In the current study, the number of non-responder samples was too small to fully assess the utility of miR-205 as a predictive biomarker. Therefore, further examination using a larger sample set is needed.

In these days, miRNA has been known to associate with tumor development, progression, and response to therapy, showing their possible use as diagnostic, prognostic and predictive biomarkers[17,23]. In veterinary medicine, it has been known to be some miRNAs as potential biomarkers for canine neoplastic disease[10]. So, in the future, miRNAs have a possibility of major biomarkers and therapeutic options.

To our knowledge, the current study provides a new evidence that miR-205 promotes radiosensitivity by modulating the E2F1-ATM pathway in CoMM cells. Our findings could lead to the development of an effective therapeutic modality to sensitize CoMM to radiotherapy.

Acknowledgments

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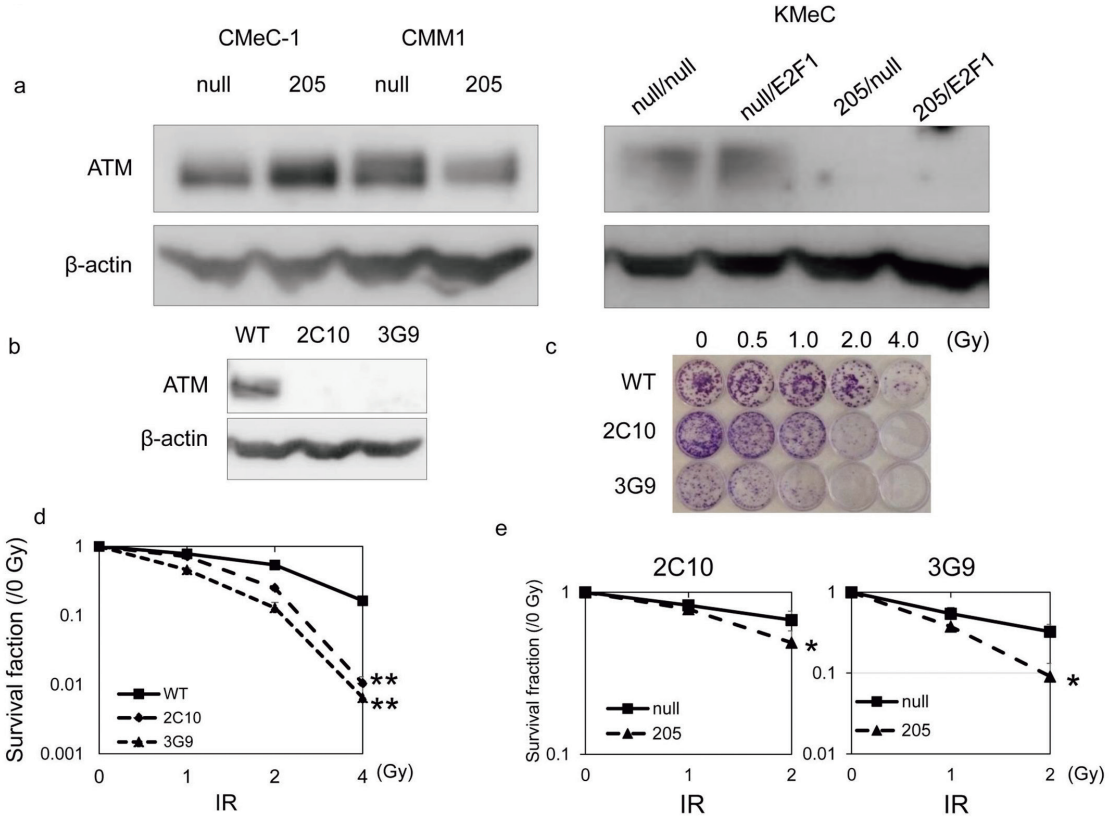

Fig. 5. Down-regulation of ATM in miR-205-overexpressed cells and enhancement of radiosensitivity by ATM knockdown. (a) The protein expression level of ATM in each cell line as determined by Western blotting analysis. (b) The expression level of ATM in wild-type and ATM-KO KMeC cells (2C10 and 3G9) as evaluated by Western blotting. (c) Results of clonogenic assay of KMeC, 2C10, and 3G9 cells after IR at the indicated dose. (d) Survival fraction for the experiment shown in "c". $^{**}P < 0.01$, for differences between WT and 2C10 or 3G9 cells. (e) Survival curves of ATM-KO cells transfected with pEP-null or pEP-hsa-miR-205 plasmid vector. $^{*}P < 0.05$, for differences between the cells transfected pEP-null (null) and pEP-hsa-miR-205 (205). Data are expressed as the mean + SD (n = 3).

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