

## Original Article

# Decreased expression levels of cell cycle regulators and matrix metalloproteinases in melanoma from RET-transgenic mice by single irradiation of non-equilibrium atmospheric pressure plasmas

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**Abstract:** Since effective therapies for melanoma with BRAF<sup>V600E</sup> mutation are being developed, interest has been shown in the development of therapies for melanoma without BRAF<sup>V600E</sup> mutation. Recently, interest has also been shown in medical application of non-equilibrium atmospheric pressure plasmas (NEAPPs). We previously suggested that repeated NEAPP irradiation to spontaneously developed benign melanocytic tumors in RFP-RET-transgenic mice (RET-mice) not only suppresses tumor growth but also prevents malignant transformation. In this study, we first confirmed that transcript expression levels of tumor growth regulators (*CyclinD1*, *D2*, *E1*, *E2*, *G2* and *PCNA* but not *CyclinG1*) and tumor invasion regulators [*Matrix metalloproteinase (MMP)-2*, *-9* and *-14* and *melanoma cell adhesion molecule (MCAM)*] in melanomas were significantly higher than those in benign melanocytic tumors in RET-mice. We then showed that transcript expression levels of *CyclinE1*, *G1* and *G2* and *MMP-2* and *-9* in melanomas from RET-mice were significantly decreased by single NEAPP irradiation, whereas transcript expression levels of *CyclinD1*, *D2*, *E2*, *PCNA*, *MCAM* and *MMP-14* were comparable in untreated and NEAPP-treated melanomas. Since no BraF<sup>V600E</sup> mutation melanomas have been found in RET-mice, our results suggest that single NEAPP irradiation is a potential therapeutic tool for melanoma without BRAF<sup>V600E</sup> mutation through modulation of the expression levels of tumor growth and invasion regulators.

**Keywords:** Melanoma, non-equilibrium atmospheric pressure plasmas, tumor growth, invasion

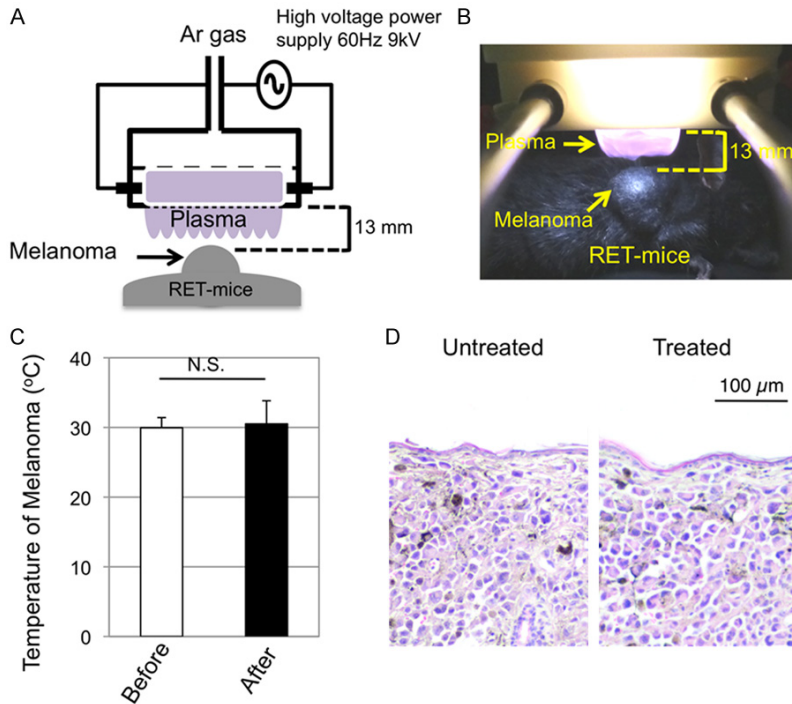
## Introduction

Melanoma, the most aggressive skin cancer, is rapidly increasing worldwide because of increased ultraviolet irradiation caused by depletion of the ozone layer. Generally, melanoma has been reported to be resistant to various therapies including X-ray irradiation. Recently, interest has been shown in a molecular target therapy for BRAF<sup>V600E</sup> mutation as a new therapy for melanoma [1]. However, therapy for melanoma without BRAF<sup>V600E</sup> mutation remains difficult. Since the results of a previous study showed that about half of melanoma

cases have no mutation of BRAF<sup>V600E</sup> [1, 2], development of a novel therapy for melanoma without BRAF<sup>V600E</sup> mutation is one of the key elements in melanoma research.

Interest has recently been shown in application of non-equilibrium atmospheric pressure plasmas (NEAPPs) for the medical field including cancer therapy. In fact, there are some reports showing anti-cancer effects of NEAPPs for various cancers including melanoma *in vitro* [3-5]. However, studies showing an *in vivo* effect of NEAPPs on transplanted cancer in animals are limited [6], and there are even fewer studies an

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**Figure 1.** Single non-equilibrium atmospheric pressure plasma (NEAPP) irradiation to melanomas. (A, B) A schema (A) and a photograph with a dark field (B) of NEAPP irradiation to melanomas in RET-mice are presented. (C) Temperatures (means  $\pm$  SD) measured by an infrared thermometer (CA380; CASON, Inc) on the melanoma surface before (n=6) and just after (n=6) single NEAPP irradiation are shown. Statistical significance was evaluated by the paired Student's t-test. (D) HE-stained microscopic appearances of melanomas in RET-mice before (Untreated) and 6 hours after (Treated) single NEAPP irradiation.

*in vivo* effect of NEAPPs on spontaneously developed cancer in animals and humans.

Multistep melanomagenesis, melanoma that has developed from a melanocytic nevus, has been reported in patients with large congenital melanocytic nevi [7]. We previously developed *RFP-RET*-transgenic mice (RET-mice) under the control of the *metallothionein-1* promoter/enhancer, in which benign melanocytic tumors and melanoma spontaneously develop with an intact immune system [8]. The entire process of melanomagenesis in RET-mice resembles that of a human melanocytic nevus [9, 10]. Since macroscopic and microscopic appearances of melanoma used in RET-mice are comparable with those of melanoma in humans, RET-mice are used worldwide as a standard animal model for melanoma [8-11].

Previous studies showed that cell cycle promoters such as *CyclinD1*, *E1* and *E2* and *PCNA* are potentially involved in the development of mel-

anoma from benign melanocytic tumors in RET-mice [12]. Matrix metalloproteinase (MMP)-2 and -9 were suggested to be associated with tumor growth [9, 13] and malignant transformation [9, 14, 15] in addition to tumor invasion and metastasis [9, 15] in tumors in RET-mice. MMP-14 and melanoma cell adhesion molecule (MCAM) have been reported to be regulators of the activity and expression of MMP-2, respectively [16, 17] and have been suggested to be associated with invasion of melanoma from RET-mice [9, 18]. Our previous study also suggested that MCAM and MMPs are involved in melanoma development from benign melanocytic tumors in RET-mice [15, 18]. Since repeated NEAPP irradiation for benign melanocytic tumors in RET-mice for 11 weeks decreased transcript expression levels

of *Cyclins*, *MCAM* and *MMPs* in our previous study, we proposed that NEAPP irradiation might suppress malignant transformation of benign melanocytic tumors in RET-mice [12, 15, 18]. Based on our previous results, we examined the effect of NEAPP irradiation on cutaneous tumors after transformation (melanoma) in RET-mice in this study.

### Materials and methods

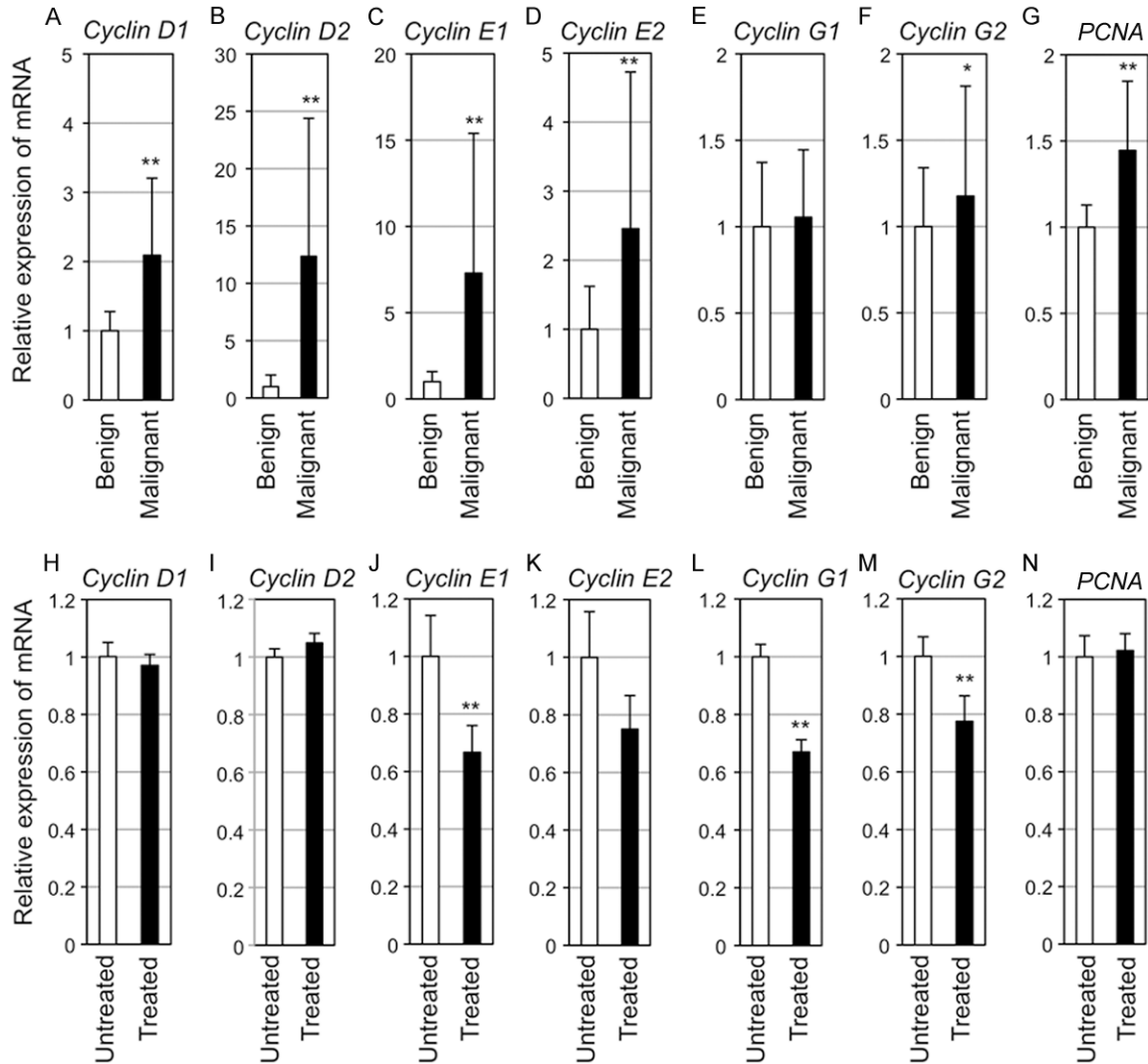
#### Tumors

Benign melanocytic tumors and melanomas in our original RET-mice [8] were used after confirming no *Braf*<sup>V600E</sup> mutation in melanomas by DNA sequence analysis according to the method described previously [19].

#### NEAPP irradiation

Transcript expression levels of cell cycle promoters, *MCAM* and *MMPs* at 6 hours after single NEAPP irradiation for 30 seconds were

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**Figure 2.** Transcript expression levels of *CyclinD1*, *CyclinD2*, *CyclinE1*, *CyclinE2*, *CyclinG1*, *CyclinG2*, and *PCNA* in tumors from RET-mice. (A-G) Relative transcript expression levels (means  $\pm$  SD) of *CyclinD1* (A), *CyclinD2* (B), *CyclinE1* (C), *CyclinE2* (D), *CyclinG1* (E), *CyclinG2* (F), and *PCNA* (G) in benign melanocytic tumors (Benign, n=6) and melanoma (Malignant, n=6) of RET-mice are presented. (H-N) Relative transcript expression levels (means  $\pm$  SD) of *CyclinD1* (H), *CyclinD2* (I), *CyclinE1* (J), *CyclinE2* (K), *CyclinG1* (L), *CyclinG2* (M), and *PCNA* (N) in NEAPP-irradiated (n=6) and unirradiated (n=5) melanomas from RET-mice are presented. The transcript expression levels were determined by quantitative PCR and normalized with the transcript expression level of *hypoxanthine ribosyltransferase* (*Hprt*) (A-N). Statistical significance was evaluated by the Mann-Whitney *U* test. \*p<0.05, \*\*p<0.01.

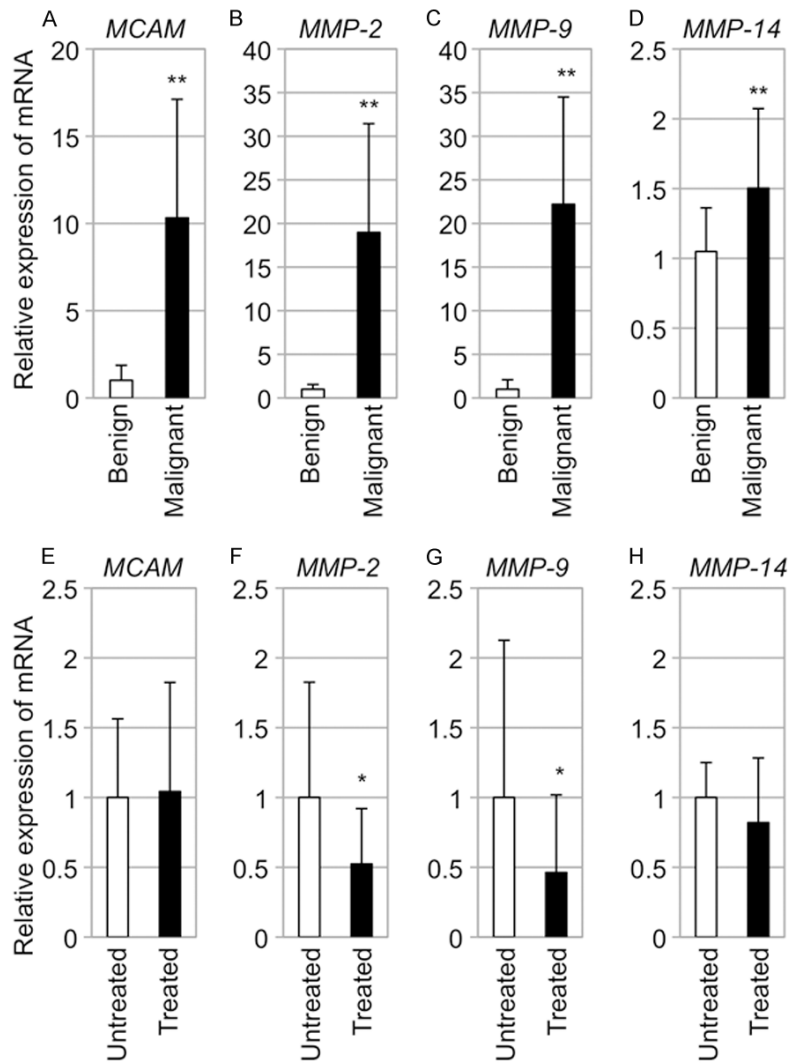
examined in pathologically diagnosed benign melanocytic tumors and melanomas from RET-mice. The method used for NEAPP irradiation (Figure 1A and 1B) in this study was the same as our previous method [12] except for the difference of single or repeated irradiation.

### Quantitative PCR (Q-PCR)

Q-PCR was performed according to the method previously described [12, 15, 18].

### Approval

The experiments using recombinant DNA were approved by the Recombination DNA Advisory Committee of Nagoya University (approval No. 12-59, 13-59, 13-76) and Chubu University (approval No. 12-04). The animal experiments were approved by the Animal Care and Use Committee of Nagoya University (approval No. 27241) and Chubu University (approval No. 2710048).



**Figure 3.** Transcript expression levels of *MCAM* and *MMPs* in melanomas from RET-mice. (A-D) Relative transcript expression levels (means  $\pm$  SD) of *MCAM* (A), *MMP-2* (B), *MMP-9* (C) and *MMP-14* (D) in melanocytic benign tumors (Benign; n=6) and melanomas (Malignant; n=6) from RET-mice. (E-H) Relative transcript expression levels (means  $\pm$  SD) of *MCAM* (E), *MMP-2* (F), *MMP-9* (G) and *MMP-14* (H) in untreated (Untreated; n=6) and NEAPP-treated (Treated; n=6) melanomas from RET-mice are presented. Statistical significance was analyzed by the Mann-Whitney *U* test. \**P*<0.05; \*\**P*<0.01.

## Results

### Effects of NEAPP irradiation on temperature and morphology of melanoma

The temperatures before and just after single NEAPP irradiation on the melanoma surface were comparable (Figure 1C). Histopathological analysis showed comparable microscopic appearances of untreated and NEAPP-treated melanoma at 6 hours after irradiation (Figure 1D).

### Effect of NEAPP irradiation on expression of regulators of the cell cycle

Constitutive expression levels of cell cycle regulatory genes, *CyclinD1*, *D2*, *E1*, *E2*, *G1*, *G2* and *PCNA*, in benign tumors and melanomas from RET-mice are shown in Figure 2A-G. Transcript expression levels of *CyclinD1*, *E1*, *E2* and *PCNA*, but not that of *CyclinG1*, in melanomas were significantly higher than those in benign melanocytic tumors, as was observed in our previous study [12]. Increased transcript expression levels of *CyclinD2* and *G2* in melanomas from RET-mice compared to those in benign melanocytic tumors were newly shown in this study.

Transcript expression levels of *CyclinE1*, *G1* and *G2* in melanoma from RET-mice were significantly decreased by single NEAPP irradiation (Figure 2J, 2L, 2M). There was no statistical difference in *CyclinD1*, *D2*, *E2* and *PCNA* transcript expression levels between untreated and NEAPP-treated melanomas (Figure 2H, 2I, 2K, 2N).

### Effect of NEAPP irradiation on expression of regulators of invasion

Constitutive transcript expression levels of invasion regulatory genes, *MCAM* and *MMP-2*, *-9* and *-14*, in benign melanocytic tumors and melanomas from RET-mice are shown in Figure 3A-D. Transcript expression levels of all of the molecules in melanomas were significantly higher than those in benign melanocytic tumors, as was observed in our previous studies [15, 18].

Transcript expression levels of *MMP-2* and *-9* in melanomas from RET-mice were significantly

decreased by single NEAPP irradiation (**Figure 2F** and **2G**). There was no statistical difference in *MCAM* and *MMP-14* transcript expression levels between untreated and NEAPP-treated melanomas (**Figure 2E** and **2H**).

### Discussion

We demonstrated for the first time that single NEAPP irradiation decreases expression levels of cell cycle regulators (*Cyclin E1*, *G1* and *G2*) and invasion regulators (*MMP-2* and *-9*) in melanoma from RET-mice with limited effects on temperature and histopathological appearance.

After repeated NEAPP irradiation to benign melanocytic tumors in original RET-mice for 11 weeks, the effect was evaluated 12 weeks after the first irradiation in our previous studies [12, 15, 18]. However, survival for 12 weeks after the first irradiation might not be easy for RET-mice with melanoma, and we therefore chose single NEAPP irradiation to melanoma in RET-mice as our first trial. To our knowledge, this is the first study showing the effect of single NEAPP irradiation on spontaneously developed melanoma in mice with an intact immune system.

We showed decreased expression levels of *CyclinE1*, *G1* and *G2* in NEAPP-treated melanomas after showing increased expression levels of *CyclinD*, *E* and *G* in melanomas from RET-mice. Since cell cycle promoters such as *CyclinE* and *G* have been suggested to be involved in the growth of tumors including melanoma in humans and mice [12, 20], our results suggest a potentially suppressive effect of single NEAPP irradiation on melanoma growth. We further showed decreased expression levels of *MMP-2* and *-9* in NEAPP-treated melanomas after showing increased expression levels of *MMP-2* and *-9* in the melanomas from RET-mice. Since the major role of *MMP-2*, *-9* and *-14* and *MCAM* has been reported to be regulation of the invasion of various cancers including melanoma in humans and mice [9, 21], our results suggest a potentially inhibitory effect of single NEAPP irradiation on melanoma invasion.

Expression levels of *CyclinD1*, *D2* and *E2*, *PCNA*, *MCAM* and *MMP-14* were comparable in untreated and NEAPP-treated melanomas.

Moreover, not only temperature but also microscopic appearance of melanoma was comparable before and after NEAPP irradiation. These results suggest that NEAPP irradiation-mediated decrease in the expression levels of regulatory molecules for tumor growth and invasion may be independent of NEAPP irradiation-mediated tissue damage.

Our results suggest that NEAPP irradiation is a novel candidate for therapy of melanoma without BRAF<sup>V600E</sup> mutation because there were no melanomas with Braf<sup>V600E</sup> mutation in RET-mice. Our results are encouraging for further studies aimed at clinical application of NEAPP irradiation for melanoma therapy.

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### Disclosure of conflict of interest

None.

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