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Original Article

Modulated expression levels of tyrosine kinases in spontaneously developed melanoma by single irradiation of non-thermal atmospheric pressure plasmas

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Abstract: Development of therapy for melanomas without $BRAF^{V600E}$ mutation, which account for about half of all melanomas, is an urgent issue because effective therapy is currently being developed for patients who have melanomas with $BRAF^{V600E}$ mutation. *RET*-transgenic mice (RET-mice) carrying the *RFP-RET* oncogene under the control of *metallothionein*-I promoter spontaneously developed skin melanomas without $Braf^{V600E}$ mutation from benign melanocytic tumors. We previously showed 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 (NEAPPs). In this study, we focused on RFP-RET, c-Ret, Epidermal growth factor receptor (Egfr), Vascular endothelial growth factor receptor 2 (Vegfr2) and c-Src kinases, which are correlated with melanoma. We first confirmed significantly increased transcript expression levels of the 5 kinases in melanomas compared to those in benign tumors in RET-mice. We then found that transcript expression levels of c-*Ret* and *Egfr*, but not those of *RFP-RET*, *Vegfr2* and c-*Src*, were significantly decreased by single irradiation of NEAPP. Since EGFR-mediated promotion of melanoma has been reported, we further focused on the mechanism of NEAPP-mediated decrease in the level of *Egfr*. Transcript expression level of *Y box protein 1* (*Ybx1*), but not those of *p53*, *Early growth factor 1* (*Egr1*), *GC-rich sequence DNA binding factor 2* (*Gcf2*) and *Kluppel-like factor 10* (*Klf10*), was significantly decreased by single irradiation of NEAPP. These results suggest that NEAPP decreased *Egfr* expression level through decrease of *Ybx1* expression. Our results indicate that NEAPP irradiation to melanoma without $BRAF^{V600E}$ mutation is a possible novel therapy.

Keywords: Non-thermal atmospheric pressure plasmas (NEAPPs), melanoma, tyrosine kinase, Epidermal growth factor receptor (Egfr), Y box protein 1 (Ybx1)

Introduction

The worldwide incidence of melanoma, the most aggressive cutaneous cancer, has recently become higher than that of any other cancer. Recent molecular-targeted therapy for melanomas with $BRAF^{V600E}$ mutation is developing in humans despite recurrence of the melanomas [1]. In fact, the prognosis of patients who have melanoma with $BRAF^{V600E}$ mutation is better than that of patients who have melanoma without $BRAF^{V600E}$ mutation [2]. Therefore, the development of a novel therapy for melanomas without $BRAF^{V600E}$ mutation, which account for about half of all melanomas, is an urgent issue.

Previous studies showed that protein tyrosine kinases (PTKs) play a crucial role in various cancers including melanoma. c-Ret/RET protein is a receptor-type PTK and is activated by dimer formation through its ligands such as a glial cell line-derived neurotrophic factor (GDNF) [3, 4].

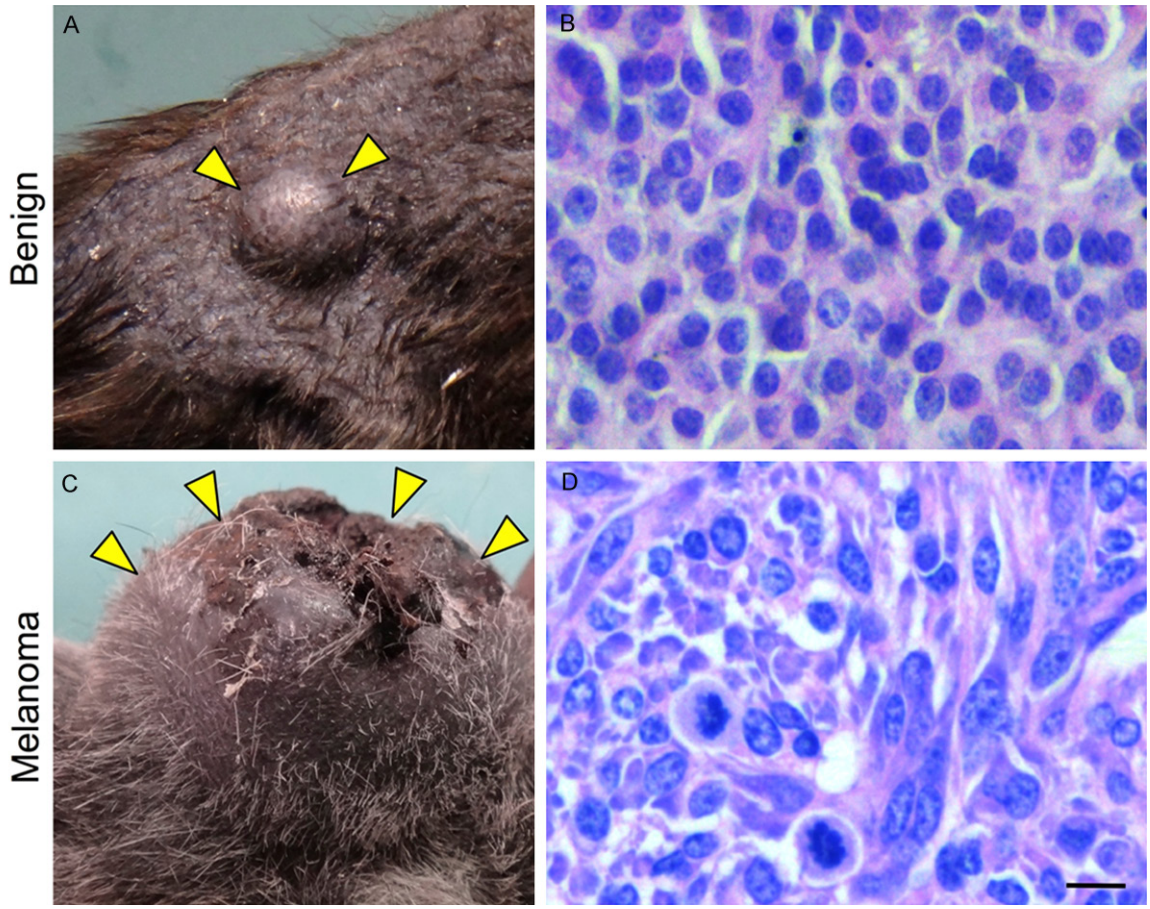

Figure 1. Macroscopic and microscopic appearances of tumors in RET-mice. (A-D) Representative macroscopic (A, C) and microscopic (B, D) appearances of a cutaneous benign melanocytic tumor (A, B) and melanoma (C, D) in RET-mice at 4 months and 12 months of age, respectively, are presented. Crusts covered the ulcer on the surface in melanoma (C) but not in the benign melanocytic tumor (A) in RET-mice. Arrowheads indicate a benign melanocytic tumor (A) and melanoma (C). Hematoxylin and eosin staining was used for histopathological analysis (C, D). Scale bar: 10 μm (D).

We have shown that anchorage-dependent growth of human melanoma cells was increased by interaction of c-RET and GDNF after confirming c-*RET* expression levels in plural human melanoma cell lines [5]. c-SRC protein, which is expressed in melanoma, is directly associated with c-RET protein and regulates the function of c-RET kinase [6]. *RFP-RET* of the hybrid gene from c-*RET* and *RFP* has an oncogenic activity and is constitutively activated without stimulation of ligands [3, 7]. *RFP-RET*-transgenic mice (RET-mice) carrying *RFP-RET* under the control of *metallothionein*-I promoter spontaneously develop benign melanocytic tumors and melanomas without $Braf^{V600E}$ mutation in a stepwise manner [7, 8]. Increased expression levels of epidermal growth factor receptor (Egfr) and vascular endothelial growth factor receptor 2 (Vegfr2) in human melanoma have been reported [9, 10]. These results suggest that RFP-RET, c-RET, Egfr, Vegfr2 and c-Src molecules are involved in the pathogenesis of melanoma.

Attention has recently been paid to medical applications of non-thermal atmospheric pressure plasmas (NEAPPs) consisting of an ionized gas. Previous studies showed anti-cancer effects of NEAPP irradiation *in vitro* [11-13]. We have also reported suppressed growth of benign melanocytic tumors in RET-mice by repeated NEAPP irradiation via decreased expression levels of cell cycle regulators and matrix metalloproteinases (MMPs) [14-16]. In our previous study, we showed an anti-cancer effect of single NEAPP irradiation to melano-

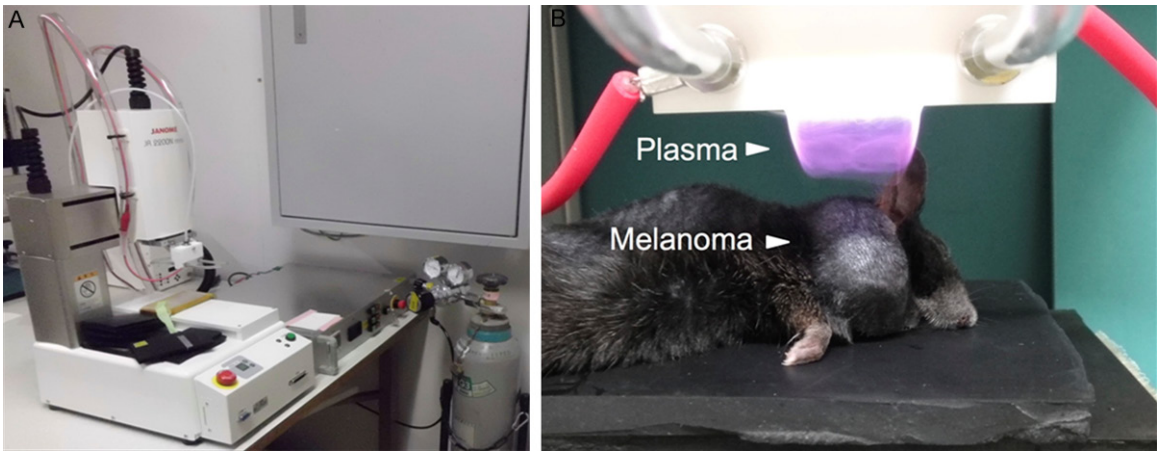

Figure 2. Non-thermal atmospheric pressure plasma (NEAPP) irradiation. (A, B) Device used for NEAPP irradiation (A) and a bright field of NEAPP irradiation to a melanoma in a RET-mouse (B) are shown. While Ar gas was provided through a tube, plasma in the main discharge region between two electrodes was excited by applying 10 kV from a 60 Hz commercial power supply. The distance between the two electrodes was 20 mm. Single irradiation for 30 sec was performed on malignant tumors from RET-mice. The RET-mouse was placed on a rubber plate to keep the distance from the plasma source at 13 mm.

mas in RET-mice via decreased levels of cell cycle regulators and MMPs [8]. In this study, we examined the effect of NEAPP irradiation on expression levels of RFP-RET, c-Ret, Egfr, Vegfr2 and c-Src in melanomas without $BRAF^{V600E}$ mutation from RET-mice.

Materials and methods

RET-mice spontaneously developed tumors in the skin

Benign melanocytic tumors (**Figure 1A**, **1B**) and melanomas (**Figure 1C**, **1D**) developed spontaneously in our original RET-mice with an intact immune system [7, 8]. Therefore, RET-mice could be a strong tool to develop various therapies for melanomas [17, 18]. Experiments using recombinant DNA were approved by the Recombination DNA Advisory Committee of Nagoya University (no. 12-39, 13-59, 13-76). The animal experiments were approved by the Animal Care and Use Committee of Nagoya University (approval no. 27241).

NEAPP irradiation to tumors in RET-mice

Benign melanocytic tumors and melanomas with or without NEAPP irradiation in RET-mice were used. The NEAPP device used in the present study is shown in **Figure 2A**. Melanomas were collected and analyzed 6 hours after single NEAPP irradiation for 30 sec for melanoma in RET-mice (**Figure 2B**) following the method previously described [8].

Quantitative PCR (Q-PCR)

After extracting total RNA from tumors of RET-mice, transcript expression levels were measured by the method described previously [14, 16]. Sequences of primers used are shown in Supplementary Table 1.

Results

Diagnosis of tumors in RET-mice

Benign melanocytic tumors and melanomas in RET-mice were preliminary selected by macroscopic observation (**Figure 1A**, **1C**). After collection of tumors with or without NEAPP irradiation, all of the tumors were histopathologically diagnosed by a trained pathologist (**Figure 1B**, **1D**). A benign melanocytic tumor consisted of uniform cells having round nuclei without mitosis (**Figure 1B**). Melanoma consisted of atypical cells having various sizes and shapes of nuclei with high mitotic activity (**Figure 1D**).

Effects of single NEAPP irradiation on expression of 5 tyrosine kinases

Transcript expression levels of *RFP-RET*, c-*Ret*, *Egfr, Vegfr2* and c-*Src* in melanomas were 2.0-fold, 1.9-fold, 9.7-fold, 1.2-fold and 2.3-fold higher than those in benign melanocytic

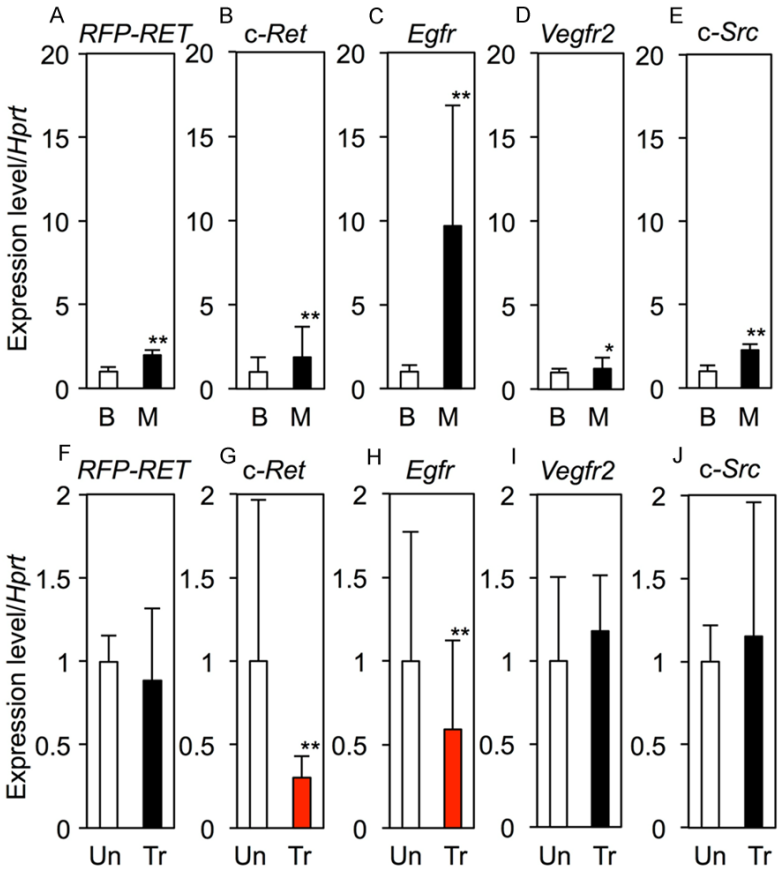

Figure 3. Transcript expression levels of *RFP-RET*, *c-Ret*, *Egfr* and *Vegfr2* in tumors from RET-mice. (A-E) Relative transcript expression levels (means ± SD) of *RFP-RET* (A, *RFP-RET*), *c-Ret* (B), *Epidermal growth factor receptor (*C, *Egfr)*, *Vascular endothelial growth factor receptor 2* (D, *Vegfr2*) and c-*Src* (E) in benign melanocytic tumors (B, n=6) and melanomas (M, n=5) from RET-mice are shown. (F-J) Relative transcript expression levels of *RFP-RET* (F), c-*Ret* (G), *Egfr* (H), *Vegfr2* (I) *and* c-*Src* (J) in untreated (Un, n=5) and NEAPP-treated (Tr, n=6) 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* (A-J, *Hprt*). Statistical significance was evaluated by Student's *t*-test. **P<0.01, *P<0.05.

tumors, respectively, in RET-mice (**Figure 3A-E**). Single NEAPP irradiation decreased expression levels of c-*Ret* and *Egfr* transcripts by 70% and 41%, respectively (**Figure 3G**, **3H**). However, the expression levels of *RFP-RET*, *Vegfr2* and c-*Src* transcripts were comparable in single NEAPP irradiated- and unirradiated-melanomas in RET-mice (**Figure 3F**, **3I**, **3J**).

Effects of NEAPP irradiation on expression of transcription factors for Egfr

Effects of single NEAPP irradiation on transcript expression levels of *Y box protein 1* (*Ybx1*), *p53, Early growth factor 1* (*Egr1*), *GC-rich sequence DNA binding factor 2* (*Gcf2*) and *Kluppel-like factor 10 (Klf-10)* in melanomas from RET-mice were further examined. Single NEAPP irradiation decreased the expression level of *Ybx1* transcript by 27% (**Figure 4A**). However, there were comparable expression levels of *p53, Egr1, Gcf2* and *Klf10* transcripts in melanomas from RET-mice despite single NEAPP irradiation (**Figure 4B-E**).

Discussion

In this study, we first confirmed higher expression levels of *RFP-RET*, c-*Ret*, *Egfr, Vegfr2* and c-*Src*, all of which were previously reported to be associated with melanoma [5, 6, 9, 10], suggesting that the kinases are also associated with melanomas in RET-mice. Then we demonstrated for the first time that expression levels of *c-Ret* and *Egfr* transcripts, but not those of *RFP-RET*, *Vegfr2* and c-*Src* transcripts, in melanomas from RET-mice were decreased by single NEAPP irradiation. Our results showed that the difference in *Egfr* expression level between benign melanocytic tumors and melanoma is the largest among the 5 kinases. Moreover, the effects of endogenous c-Ret may be replaced by the effects of the introduced RFP-RET in RET-mice. In fact, kinase activity of c-Ret was found to be lower than that of RFP-RET in RET-mice in our previous studies [3, 4, 19]. Together with a previous report showing that EGFR regulates progression and metastasis of human melanoma [20], we focused on Egfr as a representative molecule that was decreased by single NEAPP irradiation.

YBX1, p53 and EGR1 bind to the promoter of the *EGFR* gene and positively control its transcription in human osteosarcoma, breast cancer and colon cancer cells [21-23]. GCF2 and

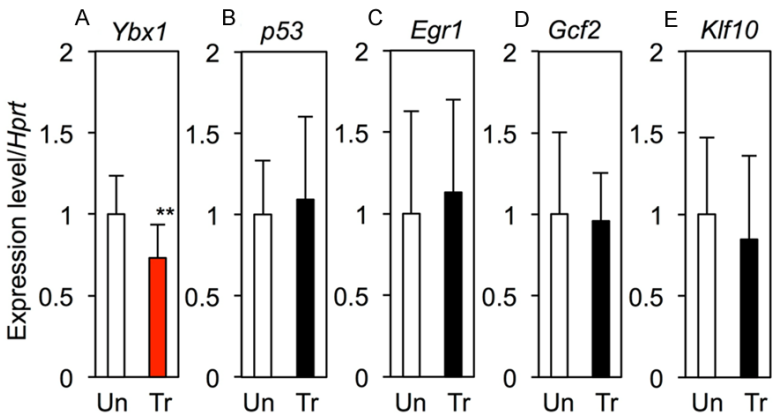

Figure 4. Transcript expression levels of *Ybx1, p53, Egr1, Gcf2 and Klf10* in irradiated melanomas from RET-mice. (A-E) Relative transcript expression levels (means ± SD) of *Y box protein 1* (A, *Ybx1*), *p53* (B), *Early growth factor 1* (C, *Egr1*), *GC-rich sequence DNA binding factor 2* (D, *Gcf2*) and *Kluppel-like factor 10* (E, *Klf10*) in untreated (Un; n=5) and NEAPP-treated (Tr, n=6) melanomas from RET-mice are presented. Statistical significance was evaluated by Student's *t*-test. **P<0.01.

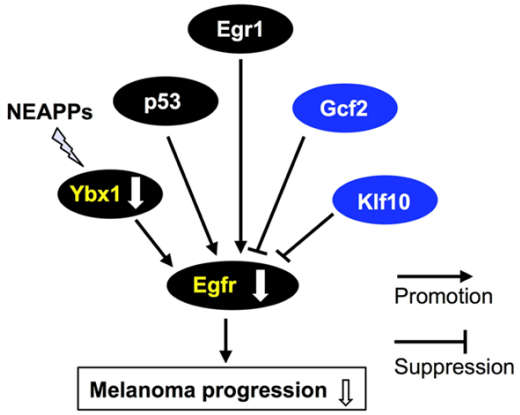

Figure 5. A schematic pathway for regulation of Egfr expression level. Y box protein 1 (Ybx1), p53 and Early growth response 1 (Egr1) progressively regulate Egfr expression level. GC-rich sequence DNA binding factor 2 (Gcf2) and Kluppel-like factor 10 (Klf10) negatively regulate Egfr expression level. Our results suggest that single irradiation of NEAPP decreases Egfr transcript expression level via suppression of Ybx1 transcript expression. Since EGFR, a regulator for melanoma progression, has been suggested to be a clinical target for melanoma therapy [20], NEAPP may be useful for melanoma therapy.

KLF10 have been reported to be transcriptional repressors of the *EGFR* gene in human melanoma cells and breast cancer cells, respectively [24, 25]. Our results further showed that single irradiation decreased the transcript expression level of only *Ybx1* among the 5 transcription factors for *Egfr*. These results suggest that single NEAPP irradiation decreases *Egfr* expression level via decreased level of *Ybx1*, a transcription factor promoting expression of *Egfr*, in melanomas without $Braf^{V600E}$ mutation from RET-mice (**Figure 5**). Since EGFR could be a clinically potential therapeutic target for melanoma [20], our results suggest that NEAPP irradiation is a novel option for therapy of melanoma without $BRAF^{V600E}$ mutation (**Figure 5**).

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Sequences of primers used in quantitative PCR

Gene	Strand	Sequence
RFP-RET	Forward	5' TCACAGGGGATGCAGTATCT 3'
RFP-RET	Reverse	5' CCTGGCTCCTCTTCACGTAG 3'
c-*Ret*	Forward	5' TGACATCAGCAAGGATCTGG 3'
c-*Ret*	Reverse	5' CCCATCGTCATACAGCAGTG 3'
Egfr	Forward	5' CAAGTGGATGGCTTTGGAAT 3'
Egfr	Reverse	5' TCCATCATAAGGCTTGGACC 3'
Vegfr2	Forward	5' TGAAATCTTTGGTGGAAGCC 3'
Vegfr2	Reverse	5' GGCCTTCCATTTCTGTACCA 3'
c-*Src*	Forward	5' TCGTGAGGGAGAGTGAGAC 3'
c-*Src*	Reverse	5' GCGGGAGGTGATGTAGAAAC 3'
Ybx1	Forward	5' GGTGCAGGAGAGCAAGGTAG 3'
Ybx1	Reverse	5' TGCCATCCTCTCTAGGCTGT 3'
p53	Forward	5' TGTCACGCTTCTCCGAAGAC 3'
p53	Reverse	5' ATCGTCCATGCAGTGAGGTG 3'
Egr1	Forward	5' GAGCGAACAACCCTATGAGC 3'
Egr1	Reverse	5' TTTGGCTGGGATAACTCGTC 3'
Gcf2	Forward	5' AACTTCCACAAGTGCCAAGG 3'
Gcf2	Reverse	5' GGAAACTTTTCTCGCCACTG 3'
Klf10	Forward	5' AGCAAGGGTCACTCCTCAGA 3'
Klf10	Reverse	5' TGAAAGGTTTTTCCCCTGTG 3'
Hprt	Forward	5' TATGTCCCCCGTTGACTGAT 3'
Hprt	Reverse	5' CTTTGCTGACCTGCTGGATT 3'