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Functional analysis of GNG2 in human malignant melanoma cells

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ABSTRACT

Background: Previous studies have revealed that heterotrimeric G protein is composed of a Gα-subunit and a Gβγ-dimer and is correlated with c-Src and AKT activities. Our recent study showed reduced G protein γ2 subunit (Gng2/GNG2) expression levels in malignant melanoma cells compared with those in benign melanocytic cells in both mice and humans. At present, however, there is no evidence showing an effect of Gng2/GNG2 alone on cancer biology.
Objective: The purpose of this study was to examine the biological significance of GNG2 in human malignant melanoma cells.
Methods: Levels of proliferation and activities of signal transduction molecules were examined in both GNG2-overexpressed and -depleted human malignant melanoma cells.
Results: Proliferation of GNG2-overexpressed SK-Mel28 human malignant melanoma cells was suppressed with decreased c-SRC and AKT activities and increased $p21^{Cip/WAF1}$ expression level *in vitro*. In contrast, proliferation of GNG2-depleted A375P human malignant melanoma cells was enhanced with increased c-SRC and AKT activities and decreased $p21^{Cip/WAF1}$ expression level *in vitro*. In the *in vivo* experiment, the mean tumor size of GNG2-overexpressed SK-Mel28 cells was less than 1/45th of that of control SK-Mel28 cells in nude mice at 95 days after inoculation.
Conclusion: We demonstrated for the first time that increased protein expression level of GNG2 alone inhibits proliferation of malignant melanoma cells *in vitro* and *in vivo*, suggesting that GNG2 could be a novel molecular target for malignant melanoma therapy.

1. Introduction

The incidence of cutaneous malignant melanoma is increasing at a greater rate than that of any other cancer [1]. Since malignant melanoma is the most serious skin cancer, malignant melanoma is a threat for human life. However, an effective therapy for malignant melanoma has not been fully established [2–4].

G protein γ2 subunit (Gng2/GNG2) is one of subunits of the Gβγ-dimer composing heterotrimeric G protein with a Gα-subunit. Heterotrimeric G protein has been reported to be involved in various biological activities including cell proliferation, differentiation, invasion and angiogenesis [5,6]. Overexpression of the Gβγ-dimer promotes cell proliferation and invasion with activation of c-SRC, FAK and PI3 kinase/AKT molecules [5–8]. These studies showed that Gβγ-dimers have tumor-promoting effects. Although there have been many Gβγ dimer-related reports, there has been very limited information on GNG2 alone, which is a component of Gβγ-dimers. To our knowledge, there is no direct evidence showing biological effects of Gng2/GNG2 alone on cancer.

We previously established RET-transgenic mice in which cutaneous benign melanocytic tumors and malignant melanomas spontaneously develop in a stepwise fashion [9,10]. We recently showed that Gng2 expression levels in malignant melanomas were significantly lower than those in benign melanocytic tumors in the mice [10–12]. We also showed that GNG2 expression levels in five human malignant melanoma cell lines were lower than those in primary normal human epithelial melanocytes (NHEM) [10–12]. These results suggest that GNG2 could be a potential biomarker for malignant melanoma. However, the biological function of GNG2 alone is still unknown.

The aim of this study was to determine the effects of GNG2 alone expression on proliferation of malignant melanoma cells in both GNG2-overexpressed and -depleted human malignant melanoma cells *in vitro* and *in vivo*.

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2. Materials and methods

2.1. Cell lines, culture conditions and proliferation assay

SK-Mel28 and A375P human malignant melanoma cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum. For proliferation assay, 1×10^5 cells were seeded in 6 cm dishes. Then the number of cells was counted each day. SRC inhibitors, PP2 and its negative control (PP3) (Wako), and an AKT inhibitor, LY294002 (Wako), were used for the proliferation assay.

2.2. Expression vectors and establishment of stable clones

Expression vector pIRES-puro3 (Invitrogen) was used for construction of the GNG2 expression vector. The human *GNG2* coding region fused with a FLAG sequence was inserted into EcoRI and BamHI sites. Empty and GNG2 expression vectors were transfected into SK-Mel28 cells, and stable cell clones were selected with 1 μg/ml puromycin (Wako). This experiment was authorized by Recombination DNA Advisory Committee (approval no. 10-08) in Chubu University.

2.3. Immunoblot and immunohistochemical analyses

Immunoblotting was performed by the method previously described [13]. Rabbit polyclonal antibodies against Gng2 (Proteintech Group), Akt (Cell Signaling), $p21^{Cip1/WAF1}$ (Abcam), and phosphorylated AKT (Cell Signaling) and mouse monoclonal antibodies against alpha-TUBULIN (SIGMA), CyclinD1 (Cell Signaling) and c-SRC (Millipore) were used as first antibodies. Immunohistochemistry was performed by the method previously described [14] with anti-Ki67 (Novocastra) and $p21^{Cip1/WAF1}$ (Abcam) antibodies. Densitometric evaluation was performed using the software program WinROOF (MITANI Corporation) as previously reported [15].

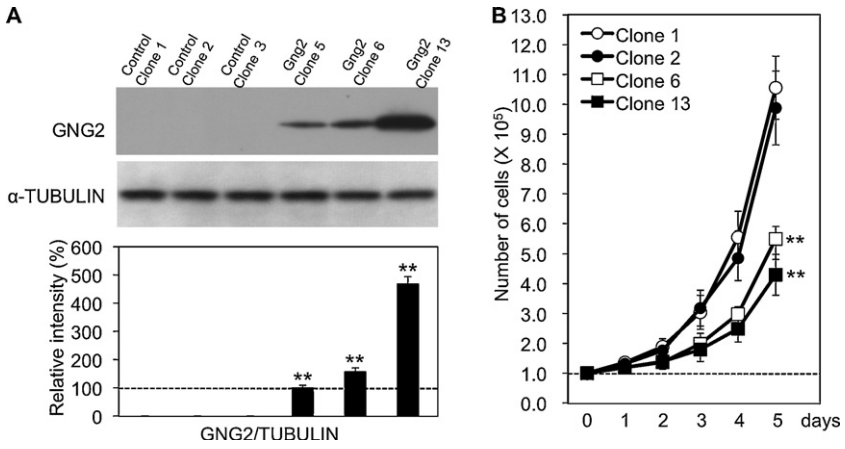

Fig. 1. Overexpression of GNG2 inhibits proliferation of SK-Mel28 cells. (A) Levels of GNG2 protein expression in stable clones of SK-Mel28 cells with empty (clones 1–3) or GNG2 expression (clones 5, 6 and 13) vectors are presented. Intensities of bands are presented as percentages (mean ± SD; $n = 3$) relative to clone 5. (B) Proliferation rates of control (clones 1 and 2) and GNG2-overexpressed (clones 6 and 13) SK-Mel28 cells are presented. 1×10^5 cells were cultured for five days and the number of cells was counted each day. Significantly different ($^{**}p < 0.01$) from the control (clone 1) by Student's *t*-test (A and B).

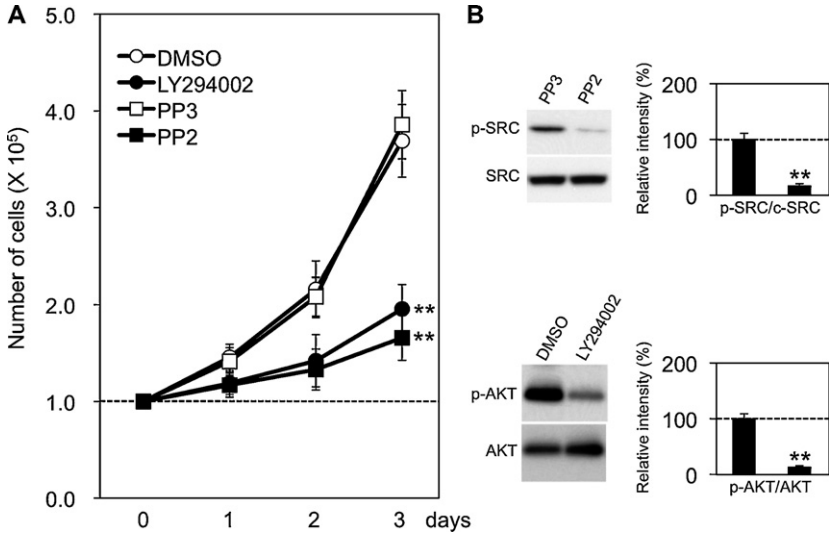

Fig. 2. Proliferation of SK-Mel28 cells is suppressed by c-SRC and AKT inhibitors. (A) *In vitro* proliferation rates (mean ± SD; $n = 3$) of SK-Mel28 cells at indicated days after treatment with DMSO (control), 10 μM of LY294002 (PI3K inhibitor), 5 μM of PP2 (SRC inhibitor) and 5 μM of PP3 (control for PP2) are presented. 1×10^5 cells were cultured for three days and the number of cells was counted each day. (B) Levels of phosphorylation and protein expression of c-SRC and AKT in the presence or absence of the inhibitor of each molecule. Intensities of bands are presented as percentages (mean ± SD; $n = 3$) relative to the control (PP3 and DMSO). Significantly different ($^{**}p < 0.01$) from the control (PP3 and DMSO) by Student's *t*-test (A and B).