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## Quantitative analysis of $\beta$ 1,6GlcNAc-branched *N*-glycans on $\beta$ 4 integrin in cutaneous squamous cell carcinoma

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### Abstract

$\alpha$ 6 $\beta$ 4 integrin plays pivotal roles in cancer progression in several types of cancers. Our previous study using *N*-glycan-manipulated cell lines demonstrated that defects in *N*-glycans or decreased  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrin suppress  $\beta$ 4 integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis. Furthermore, immunohistochemical analysis has shown that colocalization of  $\beta$ 1,6GlcNAc-branched *N*-glycans with  $\beta$ 4 integrin was observed in cutaneous squamous cell carcinoma (SCC) tissue. However, until now there has been no direct evidence that  $\beta$ 1,6GlcNAc-branched *N*-glycans are upregulated on  $\beta$ 4 integrin in cutaneous SCC. In the present study, we performed an ELISA analysis of  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrins as well as  $\beta$ 4 integrins in cell lysates from human normal skin and cutaneous SCC tissues. The SCC samples showed a 4.9- to 7.4-fold increase in the ratio of  $\beta$ 1,6GlcNAc-branched *N*-glycans to  $\beta$ 4 integrin compared with normal skin samples. These findings suggest that the addition of  $\beta$ 1,6GlcNAc-branched *N*-glycans onto  $\beta$ 4 integrin was markedly elevated in cutaneous SCC tissue compared to normal skin tissue. The value of  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrin may be useful as a diagnostic marker associated with cutaneous SCC tumor progression.

**Key words :**  $\beta$ 4 integrin, glycosylation, squamous cell carcinoma,  $\beta$ 1,6GlcNAc-branched *N*-glycan, ELISA

### Introduction

$\alpha$ 6 $\beta$ 4 integrin is a principle receptor for laminin, an extracellular matrix protein. Binding of  $\alpha$ 6 $\beta$ 4 integrin to the basement membrane protein laminin-332 plays essential roles in the formation of stable adhesion complex hemidesmosomes in the skin<sup>1-3)</sup>. The  $\alpha$ 6 $\beta$ 4 integrin is a heterodimeric transmembrane protein consisting of  $\alpha$ 6 and  $\beta$ 4 subunits.  $\beta$ 4 integrin has a unique longer cytoplasmic domain (> 1,000 amino acid residues) compared to other  $\beta$  integrin subunits (< 50 amino acid residues), and this unique domain interacts with other hemidesmosome component proteins such as plectin, BP180, and BP230<sup>4)</sup>. For this reason,  $\alpha$ 6 $\beta$ 4 integrin has long been regarded as a

component in the formation of tight adhesion. However, recent studies have shown that increased expression of  $\alpha$ 6 $\beta$ 4 integrin is correlated with tumor malignancy and poor survival of patients in several types of cancers including cutaneous squamous cell carcinoma (SCC)<sup>5-7)</sup>. Furthermore, many reports have revealed that  $\alpha$ 6 $\beta$ 4 integrin plays key roles in cancer progression by promoting cancer cell migration, invasion, proliferation, metastasis, and tumorigenesis<sup>3)</sup>. These functions of  $\alpha$ 6 $\beta$ 4 integrin are dynamically regulated by post-translational modifications, phosphorylation and *N*-glycosylation on  $\beta$ 4 integrin<sup>4,8-10)</sup>.

Glycosylation is the most common post-translational modification of proteins and has profound effects on protein folding, stability, solubility, secretion,

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transport, and interaction with other proteins<sup>11,12</sup>. Glycosylation regulates various functions of proteins and is involved in various physiological and pathological events. Protein glycosylation varies according to age, sex, and lifestyle, but overall profiles are consistent in healthy individuals<sup>12,13</sup>. In contrast, aberrant glycosylation of proteins is often associated with malignant transformation<sup>14</sup>.

$\beta$ 1,6GlcNAc-branched *N*-glycans, which are catalyzed by a member of the glycosyltransferase family, *N*-acetylglucosaminyltransferase-V (GnT-V), have been reported to be found in tumor tissues, with increased levels correlating with tumor malignancy and poor prognosis<sup>15,16</sup>. Our recent studies using cell lines in which *N*-glycan processing is genetically altered have demonstrated that defects of *N*-glycans or decreased  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrin suppress  $\beta$ 4 integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis<sup>10,17</sup>. Additionally, immunohistochemical analysis has shown that colocalization of  $\beta$ 1,6GlcNAc-branched *N*-glycans with  $\beta$ 4 integrins are observed in cutaneous SCC tissue<sup>10</sup>. However, the colocalization results in SCC tissues do not provide direct evidence that the  $\beta$ 1,6GlcNAc-branched *N*-glycans are on  $\beta$ 4 integrins because the L<sub>4</sub>-PHA lectin that was used as a probe for detecting  $\beta$ 1,6GlcNAc-branched *N*-glycans in the analysis also recognizes the  $\beta$ 1,6GlcNAc-branched *N*-glycans on other proteins. Thus, it could not be determined whether  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrin are upregulated in cutaneous SCC tissue. In the present study, we determined the expression level of  $\beta$ 1,6GlcNAc-branched *N*-glycans on  $\beta$ 4 integrin in normal human skin and cutaneous SCC tissues by ELISA using anti- $\beta$ 4 integrin antibodies (Abs) and L<sub>4</sub>-PHA lectin.

## Materials and methods

### *Sample preparation*

Normal human skin and primary cutaneous SCC samples were obtained under protocols approved by the Ethics Committee of Fukushima Medical University (number 29054), which is guided by local policy, national law, and the World Medical Association Declaration of Helsinki. We obtained one normal human skin sample and five primary SCC samples from five patients with cutaneous SCC. All patients provided written informed consent. Fresh samples were collected immediately after surgical resection and stored at  $-80^{\circ}\text{C}$ . For preparing cell lysates, the samples were lysed with a RIPA buffer [1% Nonident P40, 25

mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS] containing 5 mM EDTA, a protease inhibitor cocktail (Nacalai Tesque, #25955-24) and a phosphatase inhibitor cocktail (Nacalai Tesque, #07575-51). The samples were homogenized using a plastic pestle and then placed on ice for 20 min. After centrifugation at 15,000 rpm for 20 min at  $4^{\circ}\text{C}$  to remove debris, the resultant supernatant was collected, aliquoted into 1.5 mL tubes, and stored at  $-80^{\circ}\text{C}$  until use. The protein concentration of the cell lysate was determined using a protein assay kit (Nacalai Tesque, #29449-44).

### *Enzyme-linked immunosorbent assay (ELISA)*

For analysis of the  $\beta$ 1,6GlcNAc-branched *N*-glycan residues on  $\beta$ 4 integrin, wells of an ELISA plate (Thermo Fisher Scientific, #445101) were coated with 50  $\mu\text{L}$  of anti-rat monoclonal Ab against  $\beta$ 4 integrin (clone 439-9B, BD Transduction Laboratories, #555719, 1 : 500) in 50 mM carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, pH 9.6) overnight at  $4^{\circ}\text{C}$ . The wells were blocked with 200  $\mu\text{L}$  of 1% BSA in PBS for 1 h at  $37^{\circ}\text{C}$ , followed by incubation with 100  $\mu\text{L}$  of each sample for 1 h at  $37^{\circ}\text{C}$ . The wells were then washed three times with TBS containing 0.05% Tween 20 (TBS-T), followed by incubation for 1 h at room temperature with 100  $\mu\text{L}$  of 5  $\mu\text{g}/\text{mL}$  biotinylated-conjugated L<sub>4</sub>-PHA (Vector Laboratories, #B-1115) in TBS-T. After washing three times with TBS-T, the wells were incubated with 100  $\mu\text{L}$  of horseradish peroxidase-conjugated streptavidin in TBS-T for 1 h at room temperature. To estimate the amount of  $\beta$ 4 integrin in the cell lysates, the wells of the ELISA plate were coated with 100  $\mu\text{L}$  of each cell lysate sample overnight at  $4^{\circ}\text{C}$ . The wells were blocked with 200  $\mu\text{L}$  of 1% BSA in PBS for 1 h at  $37^{\circ}\text{C}$ , followed by incubation with 100  $\mu\text{L}$  of anti-rabbit polyclonal Ab against  $\beta$ 4 integrin (H-101, Santa Cruz Biotechnology, #sc-9090, 1 : 500) in TBS-T for 1 h at  $37^{\circ}\text{C}$ . The wells were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG Ab (Promega, #W401B, 1 : 2,000) for 1 h at room temperature. After washing the wells five times with TBS-T, color development proceeded using a TMB microwell peroxidase substrate system (KPL, #50-76-11), and the reaction was stopped by adding 1 M phosphoric acid. The color intensity was measured at 450 nm using a microplate reader (BioRad, model 680). Then, to investigate whether  $\beta$ 1,6GlcNAc-branched *N*-glycans on a  $\beta$ 4 integrin were increased by SCC development, we calculated the ratio of  $\beta$ 1,6GlcNAc-branched *N*-glycans to  $\beta$ 4 integrin by dividing the value of  $\beta$ 1,6GlcNAc-

branched *N*-glycans on β4 integrins by the value of total β4 integrins.

*Statistical analysis*

Results were given as mean ± SEM. Statistical significance was calculated among the groups using one-way ANOVA followed by a Bonferroni post-test, with GraphPad Prism Version 5.0a. *P* < 0.05 was considered statistically significant.

**Results**

Our previous immunohistochemical analysis showed that colocalization of β1,6GlcNAc-branched *N*-glycans with β4 integrins was observed in cutaneous SCC tissues<sup>10</sup>. To examine whether β1,6GlcNAc-branched *N*-glycans on β4 integrin are upregulated along with SCC development, we then evaluated the ratio of β1,6GlcNAc-branched *N*-glycans to β4 integrin in the cell lysates from normal skin and cutaneous SCC tissues. For that purpose, we developed the ELISA systems shown in Figure 1. To estimate the amount of β4 integrin, the cell lysates were coated to the wells of 96-well ELISA plates and the β4 integrin

in the cell lysates was detected using an anti-β4 integrin polyclonal Ab (H-101, Figure 1a). To measure the amount of β1,6GlcNAc-branched *N*-glycans on β4 integrins, β4 integrin in the cell lysates was captured by anti-β4 integrin monoclonal Abs (clone 439-9B) coated on plates. Then, β1,6GlcNAc-branched *N*-glycans on the captured β4 integrin were detected by L<sub>4</sub>-PHA (Figure 1b), which preferentially binds to β1,6GlcNAc-branched *N*-glycans<sup>18</sup>.

Next, we performed the modified ELISA assays using the cell lysates from normal skin and cutaneous SCC tissues to investigate whether β1,6GlcNAc-branched *N*-glycans on a β4 integrin were increased by SCC development. Then, we calculated the ratio of β1,6GlcNAc-branched *N*-glycans to β4 integrin as described in Materials and Methods. SCC samples showed a 4.9- to 7.4-fold increase in the ratio of β1,6GlcNAc-branched *N*-glycans to β4 integrin compared to normal skin samples (Figure 2). These results indicate that the addition of β1,6GlcNAc-branched *N*-glycans onto β4 integrin was markedly elevated in cutaneous SCC tissue compared to normal skin tissue.

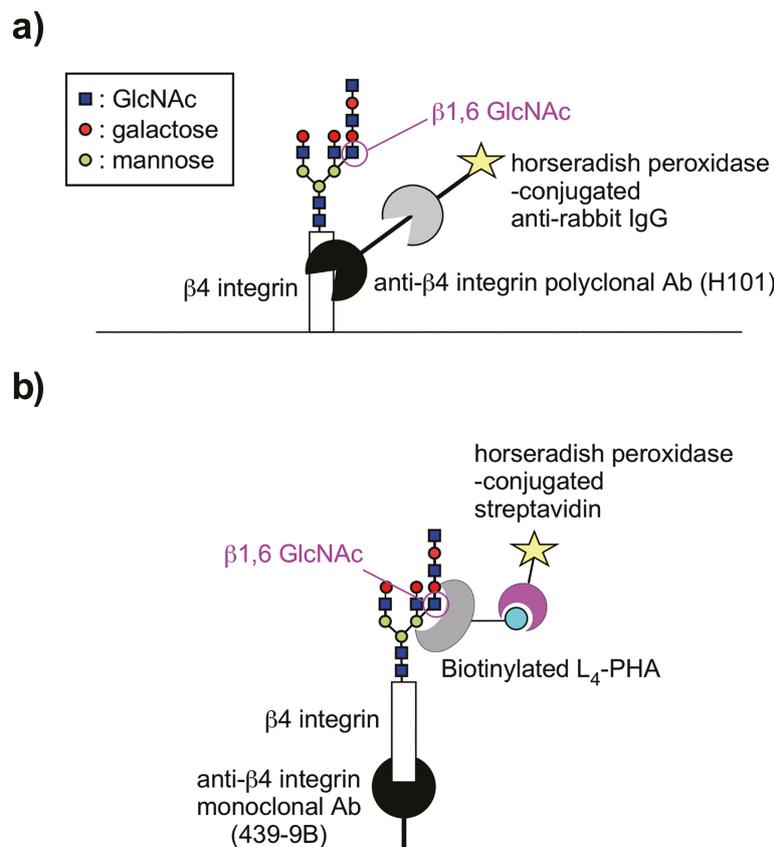


Figure 1. Schematic diagram of ELISA in this study. ELISA for detecting β4 integrins (a) and β1,6GlcNAc-branched *N*-glycans on β4 integrins (b) in cell lysates. L<sub>4</sub>-PHA preferentially binds to β1,6GlcNAc-branched *N*-glycans.

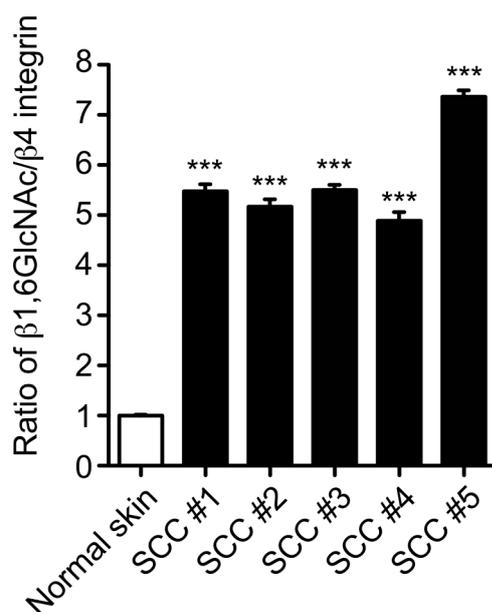


Figure 2. Increased  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin in cutaneous squamous cell carcinoma tissues. Relative amounts of  $\beta 4$  integrin and  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin in cell lysates from human normal skin ( $n = 1$ ) and cutaneous squamous cell carcinoma tissues ( $n = 5$ ) were determined by ELISA as shown in Figure 1. The graph indicates the ratio of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans to a  $\beta 4$  integrin in each sample. One-way ANOVA and Bonferroni post-test, mean  $\pm$  SEM of three independent assays conducted in triplicate. \*\*\* $P < 0.001$  vs. normal skin.

## Discussion

In the present study, the ELISA assays using cell lysates from normal skin and SCC tissues demonstrated that  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin were significantly increased in cutaneous SCC tissue compared to normal skin tissue. This finding is supported by previous immunohistochemical staining data that colocalization of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans with  $\beta 4$  integrins were observed in cutaneous SCC tissues. Other previous studies reported that defects of *N*-glycans or decreased  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin suppress  $\beta 4$  integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis<sup>10,17</sup>. Collectively, these data suggest that the upregulation of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin plays pivotal roles in cutaneous SCC development and malignant progression.

In the present study, we determined the ratio of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans to  $\beta 4$  integrin, which indicates the extent of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin, using our modified ELISA system. The relationship between this ratio and tumor malignancy was unresolved because of our small

sample size. Further studies using larger sample sizes may reveal the relationship between this value and tumor malignancy.

Overexpression of both  $\alpha 6\beta 4$  integrin and  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans has also been associated with poor prognosis in several types of cancers such as breast, colon, and esophagus<sup>5,15,19-21</sup>. Considering the current findings, it may be interesting to analyze the number of the  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin upon development of those tumors. The potential for the quantity of  $\beta 1,6\text{GlcNAc}$ -branched *N*-glycans on  $\beta 4$  integrin to become a diagnostic marker associated with tumor progression should be investigated further.

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## Author contributions

Y.K. developed the original concept. Y.K., M. Oh., Y.H., and T.Y. conceived and designed the experiments. Y.K., M.Oy., and N.K. performed the experiments. Y.K. analyzed the data. Y.K. wrote the paper.

## Conflict of interest

The authors declare no conflict of interest.

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