Quantitative analysis of β1,6GlcNAc-branched N-glycans on β4 integrin in cutaneous squamous cell carcinoma

Fukushima J. Med. Sci.,
Vol. 66, No. 3, 2020

[Original Article]

Quantitative analysis of β1,6GlcNAc-branched *N*-glycans on β4 integrin in cutaneous squamous cell carcinoma

Yoshinobu Kariya[1)], Midori Oyama[1)], Mikio Ohtsuka[2)], Nobuyuki Kikuchi[2)], Yasuhiro Hashimoto[1)] and Toshiyuki Yamamoto[2)]

[1)]Department of Biochemistry, [2)]Department of Dermatology, Fukushima Medical University School of Medicine

(Received June 17, 2020, accepted July 10, 2020)

Abstract

α6β4 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 β1,6GlcNAc-branched *N*-glycans on β4 integrin suppress β4 integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis. Furthermore, immunohistochemical analysis has shown that colocalization of β1,6GlcNAc-branched *N*-glycans with β4 integrin was observed in cutaneous squamous cell carcinoma (SCC) tissue. However, until now there has been no direct evidence that β1,6GlcNAc-branched *N*-glycans are upregulated on β4 integrin in cutaneous SCC. In the present study, we performed an ELISA analysis of β1,6GlcNAc-branched *N*-glycans on β4 integrins as well as β4 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 β1,6GlcNAc-branched *N*-glycans to β4 integrin compared with normal skin samples. These findings suggest that the addition of β1,6GlcNAc-branched *N*-glycans onto β4 integrin was markedly elevated in cutaneous SCC tissue compared to normal skin tissue. The value of β1,6GlcNAc-branched *N*-glycans on β4 integrin may be useful as a diagnostic marker associated with cutaneous SCC tumor progression.

Key words : β4 integrin, glycosylation, squamous cell carcinoma, β1,6GlcNAc-branched *N*-glycan, ELISA

Introduction

α6β4 integrin is a principle receptor for laminin, an extracellular matrix protein. Binding of α6β4 integrin to the basement membrane protein laminin-332 plays essential roles in the formation of stable adhesion complex hemidesmosomes in the skin[1-3)]. The α6β4 integrin is a heterodimeric transmembrane protein consisting of α6 and β4 subunits. β4 integrin has a unique longer cytoplasmic domain (> 1,000 amino acid residues) compared to other β integrin subunits (< 50 amino acid residues), and this unique domain interactings with other hemidesmosome component proteins such as plectin, BP180, and BP230[4)]. For this reason, α6β4 integrin has long been regarded as a component in the formation of tight adhesion. However, recent studies have shown that increased expression of α6β4 integrin is correlated with tumor malignancy and poor survival of patients in several types of cancers including cutaneous squamous cell carcinoma (SCC)[5-7)]. Furthermore, many reports have revealed that α6β4 integrin plays key roles in cancer progression by promoting cancer cell migration, invasion, proliferation, metastasis, and tumorigenesis[3)]. These functions of α6β4 integrin are dynamically regulated by post-translational modifications, phosphorylation and *N*-glycosylation on β4 integrin[4,8-10)].

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

Corresponding author : Yoshinobu Kariya, Ph.D. E-mail : kariya@fmu.ac.jp

transport, and interaction with other proteins[11,12]. 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[12,13]. In contrast, aberrant glycosylation of proteins is often associated with malignant transformation[14].

β1,6GlcNAc-branched *N*-glycans, which are catalyzed by a member of the glycsoyltransferase 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[15,16]. Our recent studies using cell lines in which *N*-glycan processing is genetically altered have demonstrated that defects of *N*-glycans or decreased β1,6GlcNAc-branched *N*-glycans on β4 integrin suppress β4 integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis[10,17]. Additionally, immunohistochemical analysis has shown that colocalization of β1,6GlcNAc-branched *N*-glycans with β4 integrins are observed in cutaneous SCC tissue[10]. However, the colocalization results in SCC tissues do not provide direct evidence that the β1,6GlcNAc-branched *N*-glycans are on β4 integrins because the L_4-PHA lectin that was used as a probe for detecting β1,6GlcNAc-branched *N*-glycans in the analysis also recognizes the β1,6GlcNAc-branched *N*-glycans on other proteins. Thus, it could not be determined whether β1,6GlcNAc-branched *N*-glycans on β4 integrin are upregulated in cutaneous SCC tissue. In the present study, we determined the expression level of β1,6GlcNAc-branched *N*-glycans on β4 integrin in normal human skin and cutaneous SCC tissues by ELISA using anti-β4 integrin antibodies (Abs) and L_4-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°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°C to remove debris, the resultant supernatant was collected, aliquoted into 1.5 mL tubes, and stored at −80°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 β1,6GlcNAc-branched *N*-glycan residues on β4 integrin, wells of an ELISA plate (Thermo Fisher Scientific, #445101) were coated with 50 μL of anti-rat monoclonal Ab against β4 integrin (clone 439-9B, BD Transduction Laboratories, #555719, 1 : 500) in 50 mM carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. The wells were blocked with 200 μL of 1% BSA in PBS for 1 h at 37°C, followed by incubation with 100 μL of each sample for 1 h at 37°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 μL of 5 μg/mL biotinylated-conjugated L_4-PHA (Vector Laboratories, #B-1115) in TBS-T. After washing three times with TBS-T, the wells were incubated with 100 μL of horseradish peroxidase-conjugated streptavidin in TBS-T for 1 h at room temperature. To estimate the amount of β4 integrin in the cell lysates, the wells of the ELISA plate were coated with 100 μL of each cell lysate sample overnight at 4°C. The wells were blocked with 200 μL of 1% BSA in PBS for 1 h at 37°C, followed by incubation with 100 μL of anti-rabbit polyclonal Ab against β4 integrin (H-101, Santa Cruz Biotechnology, #sc-9090, 1 : 500) in TBS-T for 1 h at 37°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 proceded 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 β1,6GlcNAc-branched *N*-glycans on a β4 integrin were increased by SCC development, we calculated the ratio of β1,6GlcNAc-branched *N*-glycans to β4 integrin by dividing the value of β1,6GlcNAc-

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[10]. 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_4-PHA (Figure 1b), which preferentially binds to β1,6GlcNAc-branched *N*-glycans[18].

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.

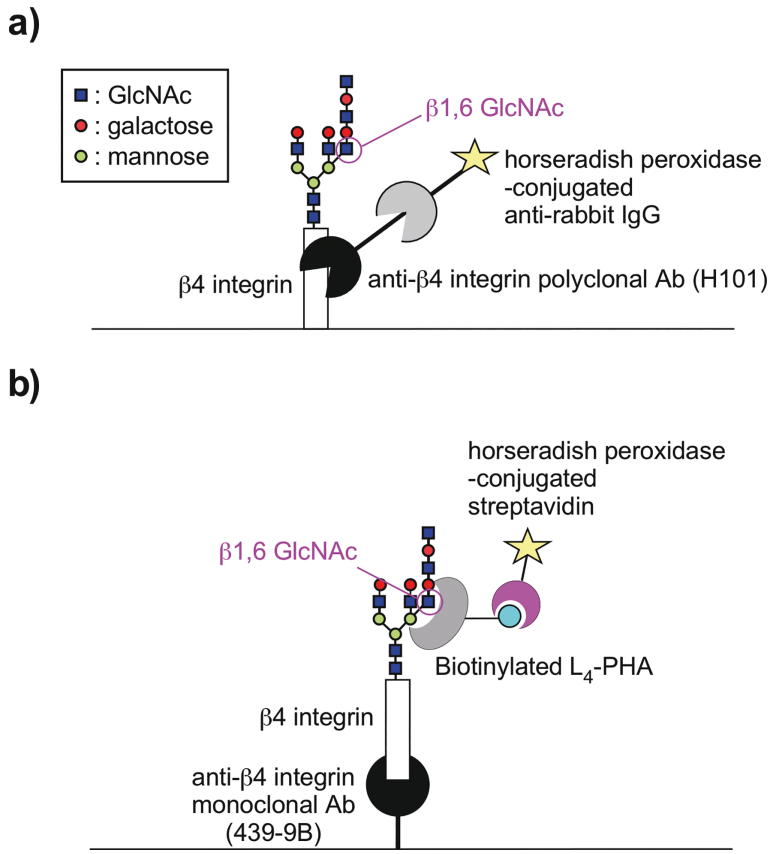

Figure 1. Schematic diagram of ELISA in this study. ELISA for detecting β4 integrins (a) and β1,6GlcNAc-branched *N*-glycans on β4 integrins (b) in cell lysates. L_4-PHA preferentially binds to β1,6GlcNAc-branched *N*-glycans.

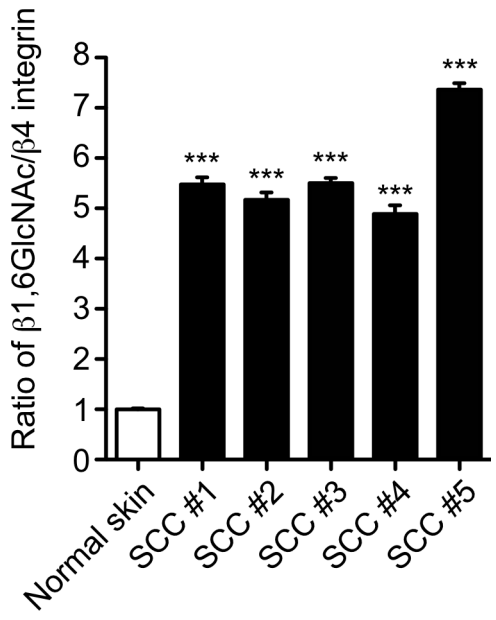

Figure 2. Increased β1,6GlcNAc-branched *N*-glycans on β4 integrin in cutaneous squamous cell carcinoma tissues. Relative amounts of β4 integrin and β1,6 GlcNAc-branched *N*-glycans on β4 integrin in cell lysates from human normal skin (n = 1) and cutaneous squamous cell carcinoma tissues (n = 5) were determined by ELISA as shown in Figure 1. The graph indicates the ratio of β1,6 GlcNAc-branched *N*-glycans to a β4 integrin in each sample. One-way ANOVA and Bonferroni post-test, mean ± 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 β1,6GlcNAc-branched *N*-glycans on β4 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 β1,6GlcNAc-branched *N*-glycans with β4 integrins were observed in cutaneous SCC tissues. Other previous studies reported that defects of *N*-glycans or decreased β1,6GlcNAc-branched *N*-glycans on β4 integrin suppress β4 integrin-mediated cancer cell adhesion, migration, invasion, and tumorigenesis[10,17]. Collectively, these data suggest that the upregulation of β1,6GlcNAc-branched *N*-glycans on β4 integrin plays pivotal roles in cutaneous SCC development and malignant progression.

In the present study, we determined the ratio of β1,6GlcNAc-branched *N*-glycans to β4 integrin, which indicates the extent of β1,6GlcNAc-branched *N*-glycans on β4 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 α6β4 integrin and β1,6GlcNAc-branched *N*-glycans has also been associated with poor prognosis in several types of cancers such as breast, colon, and esophagus[5,15,19-21]. Considering the current findings, it may be interesting to analyze the number of the β1,6GlcNAc-branched *N*-glycans on β4 integrin upon development of those tumors. The potential for the quantity of β1,6GlcNAc-branched *N*-glycans on β4 integrin to become a diagnostic marker associated with tumor progression should be investigated further.

Acknowledgements

We thank the Fukushima Medical University English editing service for their English language review. Our work was supported by grants from the Japan Society for Promotion of Science KAKENHI (25860243 and 17K08665, Y. K).

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.

References

1. Litjens SH, de Pereda JM, Sonnenberg A. Current insights into the formation and breakdown of hemidesmosomes. Trends in Cell Biol, **16** : 376-383, 2006.
2. Ko MS, Marinkovich MP. Role of dermal-epidermal basement membrane zone in skin, cancer, and developmental disorders. Dermatol Clin, **28** : 1-16, 2010.
3. Kariya Y, Kariya Y, Gu J. Laminin-332 and Integrins : Signaling Platform for Cell Adhesion and Migration and its Regulation by *N*-glycosylation. *In* : Laminins : Structure, Biological Activity and Role in Disease, **Ch. 29** : 29-51, 2013.
4. Kariya Y, Kariya Y, Gu J. Roles of Integrin α6β4 Glycosylation in Cancer. Cancers, **9**, 2017.
5. Stewart RL, O'Connor KL. Clinical significance of the integrin α6β4 in human malignancies. Lab

invest, **95** : 976-986, 2015.
6. Masugi Y, Yamazaki K, Emoto K, *et al.* Upregulation of integrin β4 promotes epithelial-mesenchymal transition and is a novel prognostic marker in pancreatic ductal adenocarcinoma. Lab invest, **95** : 308-319, 2015.
7. Dajee M, Lazarov M, Zhang JY, *et al.* NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature, **421** : 639-643, 2003.
8. Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM. Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. Cell, **91** : 949-960, 1997.
9. Dans M, Gagnoux-Palacios L, Blaikie P, Klein S, Mariotti A, Giancotti FG. Tyrosine phosphorylation of the beta 4 integrin cytoplasmic domain mediates Shc signaling to extracellular signal-regulated kinase and antagonizes formation of hemidesmosomes. J Biol Chem, **276** : 1494-1502, 2001.
10. Kariya Y, Oyama M, Hashimoto Y, Gu J, Kariya Y. β4-Integrin/PI3K Signaling Promotes Tumor Progression through the Galectin-3-*N*-Glycan Complex. Mol Cancer Res, **16** : 1024-1034, 2018.
11. Varki A. Biological roles of glycans. Glycobiology, **27** : 3-49, 2017.
12. Clerc F, Reiding KR, Jansen BC, Kammeijer GS, Bondt A, Wuhrer M. Human plasma protein *N*-glycosylation. Glycoconj J, **33** : 309-343, 2016.
13. Gornik O, Wagner J, Pucić M, Knezević A, Redzic I, Lauc G. Stability of N-glycan profiles in human plasma. Glycobiology, **19** : 1547-1553, 2009.
14. Pinho SS, Reis CA. Glycosylation in cancer : mechanisms and clinical implications. Nat Rev Cancer, **15** : 540-555, 2015.
15. Dennis JW, Laferté S. Oncodevelopmental expression of—GlcNAc beta 1-6Man alpha 1-6Man beta 1—branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. Cancer Res, **49** : 945-950, 1989.
16. Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW. Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat Med, **6** : 306-312, 2000.
17. Kariya Y, Gu J. *N*-glycosylation of β4 integrin controls the adhesion and motility of keratinocytes. PloS One, **6** : e27084, 2011.
18. Cummings RD, Kornfeld S. Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized Phaseolus vulgaris leukoagglutinating and erythroagglutinating lectins. J Biol Chem, **257** : 11230-11234, 1982.
19. Fernandes B, Sagman U, Auger M, Demetrio M, Dennis JW. Beta 1-6 branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res, **51** : 718-723, 1991.
20. Takano R, Nose M, Nishihira T, Kyogoku M. Increase of beta 1-6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro. Am J Pathol, **137** : 1007-1011, 1990.
21. Kwon J, Lee TS, Lee HW, *et al.* Integrin alpha 6 : a novel therapeutic target in esophageal squamous cell carcinoma. Int J Oncol, **43** : 1523-1530, 2013.