Original Article

Immunohistochemical Study of O-GlcNAcylation in Human Skin Tumors

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Abstract : O-linked β-N-acetylglucosamine modification is an important post-translational modification, emerging as a novel regulatory mechanism in various cellular events. Recently, several studies have shown that O-GlcNAcylation plays an essential role in human breast, lung, and colon cancers. With regard to skin cancers, the role of O-GlcNAcylation has yet to be elucidated. To investigate whether O-GlcNAcylation is linked to human skin tumor development, immunohistochemical analysis was performed to investigate the presence of O-GlcNAcylation in various skin tumors. We evaluated the levels of O-GlcNAcylation, O-GlcNAc transferase, and O-GlcNAcase in 29 benign tumors, 12 premalignant tumors, and 26 malignant tumors in skin. Compared to the benign tumors, premalignant and malignant tumors had increased patterns of O-GlcNAcylation. In addition, the O-GlcNAc transferase and O-GlcNAcase levels were higher in premalignant and malignant tumors than in benign tumors. Interestingly, O-GlcNAcase levels were significantly increased in premalignant tumors compared to benign and malignant tumors. These results suggest that O-GlcNAcylation of proteins may play an important role in the development of human skin tumors.

Keywords : Immunohistochemistry, O-GlcNAc, O-GlcNAcase, O-GlcNAc transferase, Skin neoplasm

Introduction

O-linked β-N-acetylglucosamine (O-GlcNAc) modification of proteins (O-GlcNAcylation) is emerging as an important protein regulatory mechanism, and modification by O-GlcNAcylation may affect the functionality of the target protein in a positive or negative fashion [1,2]. O-GlcNAcylation, occurring in both the cytoplasm and nucleus, is catalyzed by the enzyme O-GlcNAc transferase (OGT), which transfers a monosaccharide (GlcNAc) from UDP-GlcNAc to serine or threonine residues of the target protein. Conversely, O-GlcNAc is removed from the protein by O-GlcNAcase (OGA) (Fig. 1) [3,4]. In many ways, O-GlcNAcylation is similar to protein phosphorylation; for example the sugar can be attached or removed in response to changes in the cellular environment triggered by stress, hormones, or nutrients. This dynamic and reversible modification is emerging as a key regulator of various cellular and disease processes. Recently, it has been demonstrated that many oncogenes and tumor suppressors can be O-GlcNAcylated, implying the importance of O-GlcNAcylation as a cancer regulator [5]. It has been demonstrated that O-GlcNAcylation enhances the migration/invasion of breast cancer cells *in vitro* and lung metastasis *in vivo* [6]. O-GlcNAcylation and OGT expression were also significantly elevated in human lung and colon cancer tissues compared to the corresponding adjacent tissues [7]. Althou-
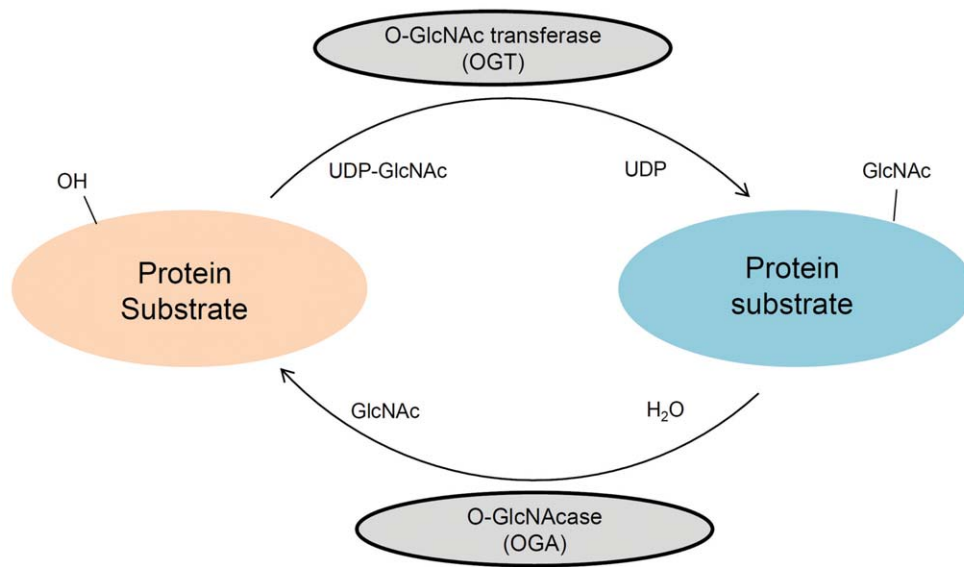
Fig. 1. The O-GlcNAcylation cycle. O-GlcNAcylation is catalyzed by the enzyme O-GlcNAc transferase (OGT), which transfers GlcNAc from UDP-GlcNAc to the target protein. Conversely, O-GlcNAc is removed from the protein by O-GlcNAcase (OGA).

Table 1. Summary of immunohistochemistry analysis

Tumor (n)	O-GlcNAc				OGT				OGA			
	−	±	+	++	−	±	+	++	−	±	+	++
Benign tumor (29)	7	8	9	5	2	8	19	0	3	21	5	0
Seborrheic keratosis (4)	0	0	1	3	0	1	3	0	0	2	2	0
Trichoepithelioma (2)	0	1	1	0	0	1	1	0	0	1	1	0
Pilomatricoma (4)	2	2	0	0	0	1	3	0	0	4	0	0
Nevus sebaceous (5)	0	1	4	0	0	0	5	0	0	5	0	0
Eccrine poroma (4)	0	0	2	2	0	2	2	0	0	3	1	0
Dermatofibroma (4)	1	2	1	0	0	0	4	0	0	3	1	0
Neurofibroma (3)	1	2	0	0	0	2	1	0	1	2	0	0
Lipoma (3)	3	0	0	0	2	1	0	0	2	1	0	0
Premalignant tumor (12)	0	0	7	5	0	2	6	4	0	3	7	2
Actinic keratosis (4)	0	0	3	1	0	0	3	1	0	0	3	1
Bowen disease (4)	0	0	2	2	0	2	1	1	0	3	1	0
Keratoacanthoma (4)	0	0	2	2	0	0	2	2	0	1	2	1
Malignant tumor (26)	0	3	7	16	0	0	15	11	2	10	13	1
Squamous cell carcinoma (16)	0	3	5	8	0	0	10	6	2	3	10	1
Basal cell carcinoma (4)	0	0	0	4	0	0	2	2	0	3	1	0
Malignant melanoma (3)	0	0	1	2	0	0	1	2	0	1	2	0
Dermatofibrosarcoma protuberans (3)	0	0	1	2	0	0	2	1	0	3	0	0

gh O-GlcNAcylation has been implicated in various cancers, O-GlcNAcylation in skin tumors has not been investigated. Using immunohistochemical analysis, we examined the overall O-GlcNAcylation patterns in several human skin tumors. We also examined the levels of OGT and OGA, two important enzymes in O-GlcNAcylation.

Materials and Methods

1. Tissue samples

All human skin samples were obtained under the written

informed consent of donors, in accordance with the ethical committee approval process of the Institutional Review Board of Chungnam National University Hospital. Immunohistochemistry analysis was performed using a monoclonal antibody against O-GlcNAc epitope in a series of 73 skin tumors (Table 1). The skin tumors included 35 benign (4 seborrheic keratosis, 2 trichoepithelioma, 4 pilomatricoma, 5 nevus sebaceous, 4 eccrine poroma, 4 dermatofibroma, 3 neurofibroma, 3 lipoma), 12 premalignant (4 actinic keratosis, 4 Bowen disease, 4 keratoacanthoma) and 26 malignant tumors (16 squamous cell carcinoma, 4 basal cell carcinoma, 3 melanoma, 3 dermatofibrosarcoma protuberans). Pilomatricoma, dermatofibroma, neurofibroma, lipoma, Bowen disease, malignant melanoma and dermatofibrosarcoma protuberans were obtained from the trunk. Seborrheic keratosis, trichoepithelioma, eccrine poroma, actinic keratosis, keratoacanthoma, squamous cell carcinoma and basal cell carcinoma were obtained from face. Nevus sebaceous was obtained from scalp. We also used 3 normal skin samples for comparative analysis.

2. Immunohistochemistry

Immunohistochemical staining was carried out on 5 μm sections from paraffin-embedded formalin-fixed blocks. The antibodies used in this study were as follows; monoclonal mouse antibody against O-GlcNAc epitope, RL2 (Abcam, Cambridge, MA), polyclonal rabbit antibody against OGT (Sigma-Aldrich, St. Louis, MO), and polyclonal rabbit antibody against OGA (Sigma-Aldrich, St.

Louis, MO). The sections were deparaffinized in xylene and then rehydrated through an alcohol series. Antigens were retrieved by pressure-cooking in citrate buffer (10 mM citric acid, pH 6.0) for 4 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 2% hydrogen peroxide for 30 minutes at room temperature. The section was incubated with primary antibody for overnight, followed by incubation with appropriate secondary antibody for 1 h, and then visualized with Chemmate envision detection kit (Dako, Carpinteria, CA). Slides were counter stained with Hematoxylin. After immunohistochemical staining, 2 dermatologists and 1 pathologist evaluated all the slides. The results of immunohistochemical staining were classified into four categories, according to the degree of intensity as follows; $++$ ($>50\%$ of the tumor cells), $+$ ($10 \sim 50\%$), $\pm$ ($5 \sim 10\%$), and $-$ ($<5\%$) and the tumors more than $+$ was considered positively stained skin specimen.

3. Statistical analysis

Data were evaluated statistically using one-way ANOVA using SPSS software (ver 22.0). Statistical significance was set at $p < 0.05$.

Results

The overall pattern of O-GlcNAcylation may be determined immunohistochemically using an antibody specific
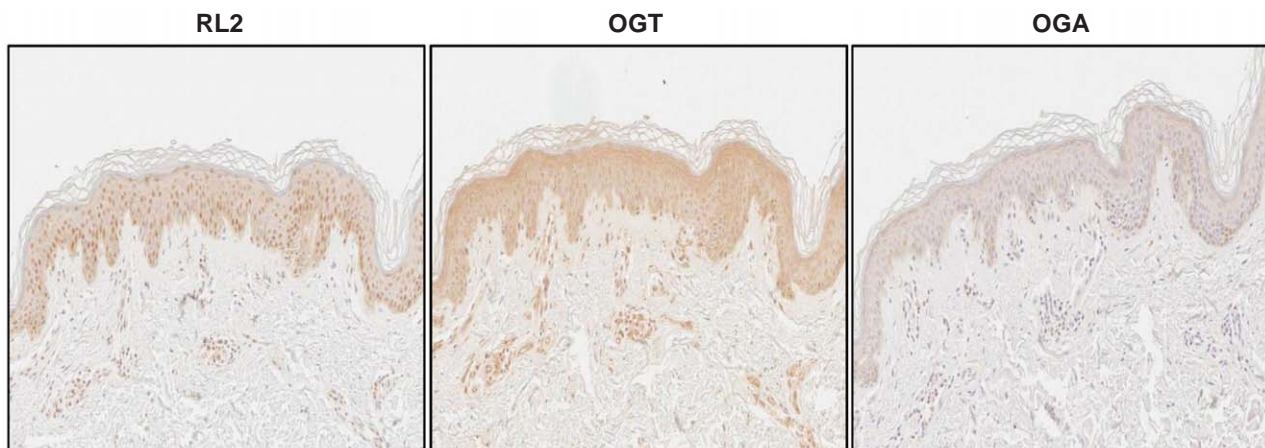

Fig. 2. Immunohistochemical analysis of O-GlcNAcylation in the epidermis. Normal skin tissues were incubated with anti-O-GlcNAc antibody (RL2), anti-O-GlcNAc transferase (OGT) antibody, and anti-O-GlcNAcase (OGA) antibody. RL2-positive staining is slightly decreased in the upper layer. OGT is observed in all layers, and intense OGA staining is seen in the upper layer (200×).

to the O-GlcNAc epitope. To investigate O-GlcNAcylation in human skin tumors, immunohistochemical analysis was performed using paraffin-embedded tissue specimens with Anti-O-GlcNAc antibody (RL2) [8], which bind to the O-GlcNAc moiety regardless of the target protein. A total of 67 skin tumors were included in the analysis and categorized as benign, premalignant, or malignant tumors. The results of the immunohistochemical analysis are summarized in Table 1. In the epidermis, positive staining was observed for O-GlcNAcylation in whole layers both in nucleus and cytoplasm. However, the staining intensity decreased towards the upper layers, indicating that O-GlcNAcylation was lower in differentiated keratinocytes (Fig. 2). To investigate the levels of the enzymes regulating O-GlcNAcylation (OGT and OGA), immunohistochemical analysis was performed. OGT expression was observed in all of the epidermal layers and OGA levels were increased in the upper layers especially in granular
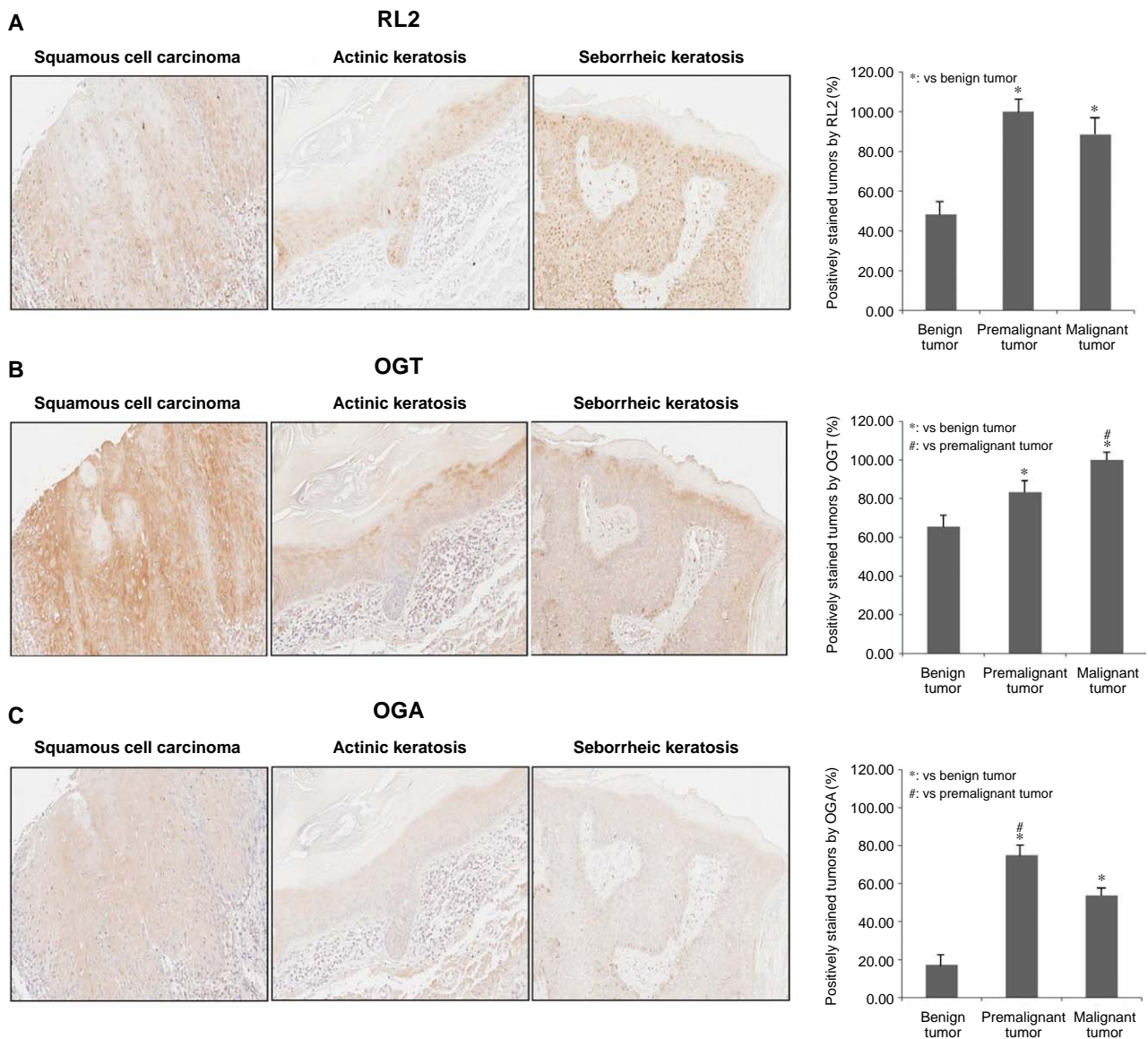

Fig. 3. Immunohistochemical analysis of O-GlcNAcylation in skin tumors. Skin tumors were categorized as benign, premalignant, or malignant and stained with (A) anti-O-GlcNAc antibody (RL2) (B) anti- O-GlcNAc transferase (OGT) antibody, and (C) anti-O-GlcNAcase (OGA) antibody. The upper panel represents a benign tumor (neurofibroma), the middle panel represents a premalignant tumor (actinic keratosis), and the lower panel represents a malignant tumor (squamous cell carcinoma) (200×). Measurements were conducted in triplicate and data are expressed as the mean±standard deviation (p<0.05).

layers (Fig. 2). Immunostaining with anti-O-GlcNAc anti-body (RL2) revealed that the staining intensity was generally higher in premalignant and malignant tumors than in benign tumors (*p<0.05), suggesting that O-GlcNAcylation was higher in more advanced tumors (Fig. 3A). Consistent with the data obtained using the RL2 antibody, immunohistochemical investigation of OGT revealed that OGT levels were generally higher in premalignant and malignant tumors compared to that of benign tumors (*p<0.05) (Fig. 3B). OGA levels were increased in premalignant and malignant tumors compared to benign tumors. Interestingly, OGA levels were significantly higher in premalignant tumors than in malignant tumors (#p<0.05) (Fig. 3C).

Discussion

O-GlcNAc modification of proteins is linked to cancer development and progression in various tissues, including lung, breast, prostate, colon, liver, and pancreas [9-13]. In lung and colon cancer, immunohistochemical studies have indicated that O-GlcNAcylation and OGT expression are significantly higher in cancer tissues compared to corresponding adjacent tissues. In breast cancer, immunohistochemical analysis has demonstrated that the global O-GlcNAcylation level significantly elevated in cancer tissues compared to corresponding adjacent tissue. O-GlcNAcylation in the metastatic lymph nodes is significantly higher than in corresponding primary tumor tissues [14]. However, the expression and functional roles of O-GlcNAcylation and associated enzymes in skin tumors have yet to be determined.

In this study, in normal epidermis, OGT was observed in all epidermal layers, and OGA levels were increased in the upper layers. These results suggest that O-GlcNAcylation may be an important regulatory mechanism during keratinocyte differentiation, and a decrease in the overall level of O-GlcNAcylation may be due to increased expression of OGA, rather than OGT. Many proteins have been identified as the target for OGT including cytoskeletal proteins, metabolic enzymes, signaling molecules, and transcription factors [14,15]. In addition, recent studies have suggested that O-GlcNAcylation is linked to various diseases such as type 2 diabetes mellitus and Alzheimer's disease [16].

It has also been suggested that increased O-GlcNAcylation may be due to elevated UDP-GlcNAc concentrations, reduced expression and catalytic activity of OGA, and/or increased OGT expression and activity [7]. In this study, increased O-GlcNAcylation was detected in premalignant and malignant skin tumors, coinciding with higher levels of OGT expression. Previous studies have demonstrated that OGT expression is markedly increased in various cancer tissues compared to corresponding adjacent tissues, while OGA levels are reduced in cancer cells [17]. An overall increase in O-GlcNAcylation is suggested to be a general phenomenon in cancer, although the precise relationship between O-GlcNAcylation and cancer remains to be elucidated.

Also, we found that OGA levels were increased in premalignant and malignant tumors compared to benign tumors, and were significantly increased in premalignant tumors compared to malignant tumors. However, the exact mechanism for the abnormal O-GlNAc modification and its regulation by OGT and OGA in premalignant and malignant skin tumors requires further investigation.

To date, more than 500 proteins have been identified to be O-GlcNAcylated. One important target protein is p53, a well-known tumor-suppressor protein. O-GlcNAcylation of p53 leads to a conformational change that prevents the phosphorylation of serine residues in close proximity to O-GlcNAc-attached serine residues [18]. As p53 is a cell cycle regulator, and changes to its activity are directly linked to cancer development and progression, it may be speculated that increased O-GlcNAc modification of this tumor-suppressor protein may contribute to the development of skin tumors. However, the precise link between tumor suppressors and O-GlcNAcylation in skin tumors is not known.

O-GlcNAc and OGT may be not only important in pathogenesis of cancers but as well as therapy of cancers. Reduction of O-GlcNAcation through RNA interference of OGT in breast cancer cells leads to inhibition of tumor growth both in vitro and in vivo [9]. And, in hepatocellular carcinoma, in vitro assays demonstrated that O-GlcNAcylation plays an important role in migration, invasion, and viability of hepatocellular carcinoma cells [12]. Therefore, O-GlcNAcylation and its related enzymes OGT or OGA can be the novel therapeutic targets for tumors. In conclusion, the results of this study demonstrate that overall O-GlcNAcylation, OGT, and OGA were increased in premalignant and

malignant skin tumors compared to benign tumors, contributing to a better understanding of the relationship between O-GlcNAcylation and skin tumors. Further studies are required to compare the functional role of O-GlcNAcylation between premalignant and malignant skin tumors.

Acknowledgements

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피부종양에서의 O- GlcNAcylation의 면역조직화학적 고찰

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간추림 : 단백질 전사 후 변형 (posttranslational modification)은 단백질 기능을 조절하는 기전의 하나로, 당화 (gycosylation), 아세틸화 (acetylation), 메틸화 (methylation) 등을 포함한다. O-linked β-N-acetylglucosamine (O-GlcNAc)에 의한 단백질 전사 후 변형은 많은 세포 반응에 관여하는 조절 기전으로 최근 연구가 많이 진행되고 있다. 특히, O-GlcNAc에 의한 단백질 전사 후 변형 (O-GlcNAcylation)은 유방암, 폐암 및 대장암 등과 같은 암 질환의 발병에 관여함이 보고되었다. 그러나 이러한 O-GlcNAcylation이 피부종양에 어떠한 영향을 미치는지 잘 밝혀져 있지 않다. 본 연구에서는 다양한 피부종양을 대상으로 하여 O-GlcNAcylation이 어떻게 일어나는지 면역 조직화학을 통해 살펴보았다. 그 결과, 양성종양에 비해 암전구증 및 악성종양에서 전체적으로 O-GlcNAcylation 이 증가해 있었다. 또한 O-GlcNAcylation을 담당하는 효소인 O-GlcNAc transferase (OGT) 및 O-GlcNAc을 제거 하는 효소인 O-GlcNAcase (OGA) 발현 또한 양성종양에 비해 암전구증 및 악성종양에서 증가해 있었다. 이러한 연구 결과는 O-GlcNAcylation이 여러 피부종양의 발병에 관여함을 시사한다.

찾아보기 낱말 : 면역조직화학염색, O-GlcNAc, O-GlcNAcase, O-GlcNAc transferase, 피부 종양

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