Elevation of nucleocytoplasmic ß-*N*-acetylglucosaminidase (*O*-GlcNAcase) activity in thyroid cancers

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Abstract. Single *N*-acetylglucosamine residues attached by *O*-linkage to serine or threonine (*O*-GlcNAc) are an abundant, dynamic and inducible post-translational modification of cytoplasmic and nuclear proteins. This study analyzes the activity of the enzyme involved in the removal of these sugar residues, i.e. ß-*N*-acetylglucosaminidase (*O*-GlcNAcase) as well as the level of *O*-GlcNAc in benign and malignant thyroid lesions. Our results demonstrate increased activity of the enzyme in thyroid cancers in comparison to non-neoplastic lesions and adenomas. *O*-GlcNAc-modified proteins in thyroid cells have a predominantly nuclear distribution and are more abundant in non-neoplastic lesions than in tumors. Understanding the aberrant *O*-GlcNAc metabolism in thyroid cancer cells may be helpful for developing new diagnostic or treatment methods.

Introduction

Two types of *N*-acetyl-ß-hexosaminidases were found in eukaryotic cells, i.e. acidic, lysosomal enzymes and neutral, cytosolic ones (1). Lysosomal enzymes are exoglycosidases that remove terminal ß-glycosidically bound *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) residues from a number of glycoconjugates (2). This group of hexosaminidases comprises the A, B, I, P and S isozymes (1-4). Two major lysosomal hexosaminidase isozymes, A and B were extensively studied because dysfunction of either of them results in the accumulation of gangliosides and other glycoconjugates within the lysosome causing the neurodegenerative disorders known as Tay-Sachs and Sandhoff disease (2,5). Moreover, the enhancement of ß-hexosaminidase degradative activity in different human cancer tissues and in serum or urine of patients with cancers is well documented (6-15).

The second type of *N*-acetyl-ß-hexosaminidases consists of cytosolic, neutral hexosaminidases, i.e. the GlcNAc-specific glucosaminidase (hexosaminidase C) and the GalNAc-specific galactosaminidase (hexosaminidase D) (1,16,17). The substrates for nonlysosomal hexosaminidases have been unknown for a long time. It changed when modification of nucleocytoplasmic proteins by single *O*-linked *N*-acetyl-ß-glucosamine (*O*-GlcNAc) was discovered. Dong and Hart (1) in 1994 partially purified and characterized enzyme from rat spleen cytosol responsible for the removal of *O*-GlcNAc from cytosolic and nuclear proteins and termed it *O*-GlcNAcase. Cloning and more detailed characterization of the enzyme in the next couple of years suggest that *O*-GlcNAcase most likely represents the previously described hexosaminidase C activity (18,19). Unlike acidic hexosaminidases in lysosomes, *O*-GlcNAcase has a neutral pH optimum, nucleocytoplasmic distribution and it is not inhibited by GalNAc (18,19).

The dynamic and inducible nature of *O*-GlcNAc suggests that this modification is more similar to phosphorylation than to classical glycosylation (20). Changes in *O*-GlcNAc levels on key proteins affect regulation of biological processes such as cell cycle, insulin signaling and protein degradation. Perturbations in the cellular *O*-GlcNAc levels have been implicated in the etiology of type II diabetes and neurodegenerative diseases, i.e. Parkinson's disease and Alzheimer's disease (21). Moreover, *O*-GlcNAc is suggested to play a role in cancer. The changes in gene expression, proliferation rate, morphology, and energy utilization observed in neoplastic cells may be associated with changes in *O*-GlcNAc metabolism (22). There is still a growing number of *O*-GlcNAc-modified proteins that play important roles in cell growth and proliferation. C-Myc, the product of the c-*myc* proto-oncogene that regulates gene transcription in cell proliferation, cell differentiation, apoptosis and metabolism is *O*-glycosylated and the major *O*-GlcNAc attachament site (Thr 58) is a mutational hot-spot for Burkitt's lymphoma (23,24). In addition, many other oncogene and tumor suppressor gene products such as c-fos, c-jun, simian virus 40 T-antigen, p53 and pRB were shown to be modified by *O*-GlcNAc (20).

The suggestion that dysregulation of *O*-GlcNAc metabolism is likely to be involved in cancer and information on increased ß-hexosaminidase activity in some tumors

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prompted us to examine for the first time *O*-GlcNAcase activity in human thyroid lesions.

Materials and methods

Surgical specimens. Surgical specimens were obtained from 49 patients (10 males and 39 females), who underwent surgery for nodular thyroid disease. The studies were performed on 18 specimens of non-neoplastic lesions (nodular goiters, hyperplasia nodularis), 7 cases of follicular adenomas, 5 follicular, 17 papillary, and 2 anaplastic carcinomas. All cases of follicular carcinoma showed the presence of capsular and vascular invasions but there were no widely invasive cases. The papillary carcinoma cases were classified as stage I and II ($T_{1-2}N_{0-1}M_0$) according to TNM staging system accepted by the International Union Against Cancer (UICC; 2002).

Tissue homogenization. Tissue samples were homogenized for 3 min in 5 volumes of homogenization buffer (20 mM Tris-HCl pH 6.8, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 1 mg/ml aprotinin). The homogenate was centrifuged at 10000 x g for 20 min. The pellet was discarded and the supernatant was used for the hexosaminidase and *O*-GlcNAcase activity assays.

Lysosomal hexosaminidase assay. The lysosomal hexosaminidase assay was performed at pH 5.3 using pNP-GlcNAc (4-nitrophenyl *N*-acetyl-D-glucosaminide) (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. Diluted supernatant samples (100 μl) containing 30 μg of protein were added to 100 μl of 2 mM p-NP-GlcNAc in citric acid buffer and incubated for 1 h at 37˚C. Activity assay was performed in triplicate. At the end of the incubation, the reaction was stopped with the addition of 0.8 ml of 0.5 M sodium carbonate. Color was measured spectrophotometrically at 400 nm with blanks routinely subtracted. Activity is reported as nanomoles of *p*-nitrophenol cleaved per minute of incubation per milligram of proteins.

O-GlcNAcase activity assay. *O*-GlcNAcase activity was measured in supernatant samples of thyroid lesions in two different ways. In the first case, the assay was based on the method described by Slawson *et al* (25). Two supernatant samples sets were prepared. In the first set, 20 μl of supernatant samples were added to 80 μl of 0.2 M MES buffer [2-(N-morpholino)ethanesulfonic acid] (pH 6.45). The second set of samples were assayed in the presence of streptozotocin (STZ) an *O*-GlcNAcase inhibitor. The samples were mixed with 20 mM STZ in MES buffer in proportion 1:2, incubated at 4˚C for 15 min and the volume was completed to 100 μl. Then, the samples were treated as in the hexosaminidase activity assay and the only exception was that the substrate was diluted in MES buffer instead of citric buffer. The activity of the STZ samples was subtracted from the non-treated samples and the difference comprised the *O*-GlcNAcase activity.

O-GlcNAcase activity was assayed also in the presence of 100 mM ß-*N*-acetyl-galactosamine. The supernatant samples were mixed with 200 mM ß-*N*-acetylgalactosamine in proportion 1:1 and incubated for 15 min at 37˚C. Then 100 μl of 2 mM p-NP-GlcNAc in MES buffer was added and the samples were incubated at 37˚C for 1 h. The reaction was stopped with the addition of sodium carbonate.

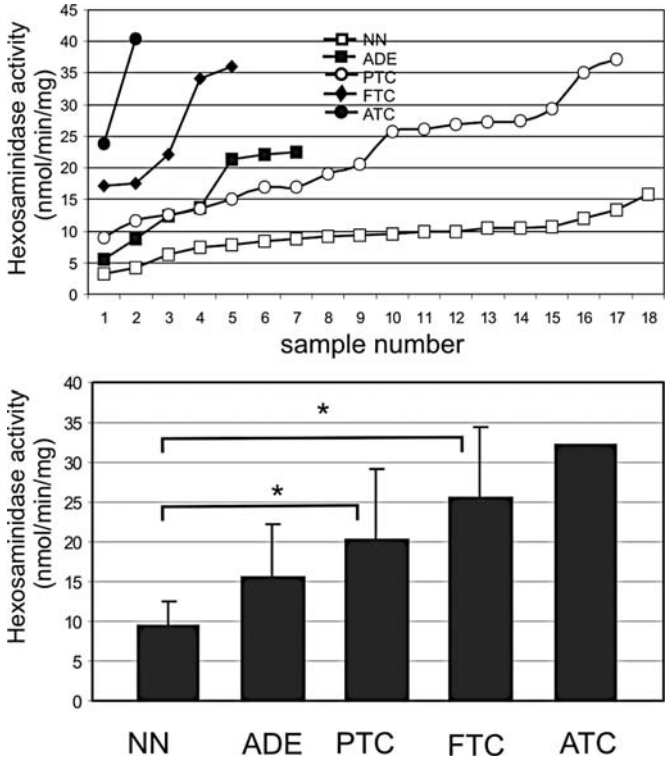

Figure 1. Hexosaminidase activity in thyroid pathological specimens. The results on upper diagram are shown as the means from triplicate determinations. The mean value for each group is shown on the lower diagram. NN, non-neoplastic specimens; ADE, adenoma; PTC, papillary carcinoma; FTC, follicular carcinoma; ATC, anaplastic carcinoma. Error bars represent standard deviation.

Isolation of cytoplasmic and nuclear fractions. The nuclei were isolated from thyroid specimens by sucrose method using 0.5% Triton X-100 to remove membrane ghost and 1 mM PMSF to inhibit protease activity. The supernatants obtained after sedimentation of nuclei from homogenates of the examined samples in 0.25 M sucrose, 5 mM $MgCl_2$, 0.8 mM KH_2PO_4 (pH 6.7) were considered as cytoplasmic fraction.

Western blot analysis of O-GlcNAc modified proteins. The samples (50 μg/lane) of cytosolic and nuclear proteins of different types of thyroid lesions were resolved by 8% SDS-PAGE and electroblotted onto Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The blots were treated for 1 h with blocking reagents (0.5% caseine in Tris buffered saline-TBS), then incubated with monoclonal antibodies specific for a single *O*-GlcNAc, i.e. RL2 (Affinity Bio-Reagents, CO, USA) or CTD110.6 (Covance, Princeton, NJ, USA). RL2 antibody was used in 1:1000 dilution (1 h incubation, room temperature) and CTD 110.6 was diluted 1:2000 (overnight incubation at 4˚C). After being washed three times with TBS, the membranes were incubated with goat anti-mouse IgG (RL2) and IgM (CTD 110.6) conjugated

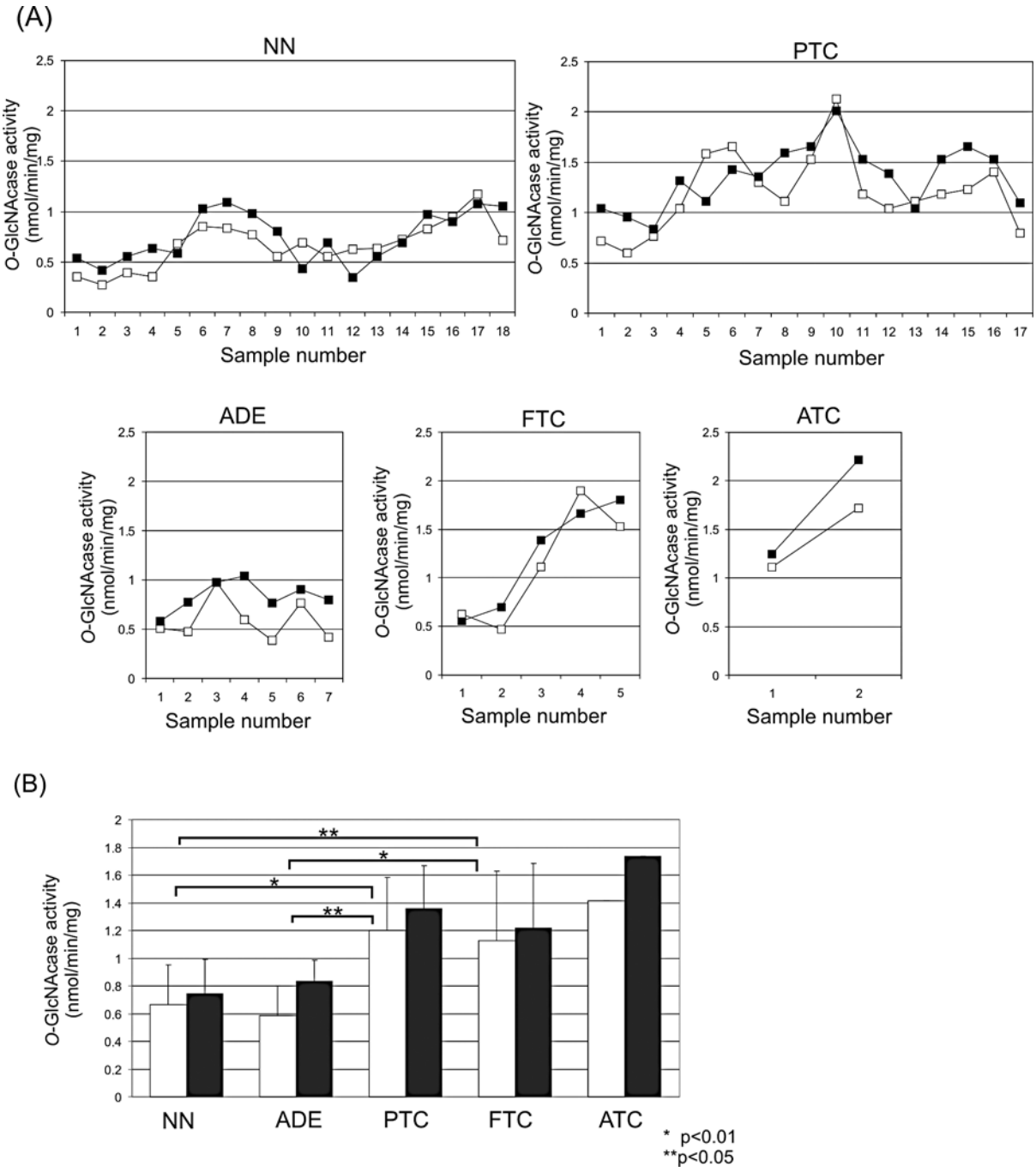

Figure 2. *O*-GlcNAcase activity in thyroid pathological specimens. Activity was measured with or without streptozotocine (STZ). The activity of the STZ samples were subtracted from the non-treated samples and the differences comprised the *O*-GlcNAcase activity (open squares). The *O*-GlcNAcase activity was measured also in the presence of 100 mM GalNAc (filled squares). The results are shown as the means from triplicate determinations (A). The mean value for each group is shown (B). NN, non-neoplastic specimens; ADE, adenoma; PTC, papillary carcinoma; FTC, follicular carcinoma; ATC, anaplastic carcinoma. Error bars represent standard deviation.

with horseradish peroxidase, added in a 1:5000 dilution. The membranes were again washed several times with TBS and proteins bearing *O*-GlcNAc were visualized on X-ray film by enhanced chemiluminescence method.

Statistical analysis. Statistical analysis was performed using the computer program Statistica version 8 (StatSoft, Inc.). The groups were compared using Mann-Whitney rank sum test. $P<0.05$ was considered statistically significant.

Results

The hexosaminidase and *O*-GlcNAcase activity was determined in homogenates of different pathological thyroid specimens by a colorimetric method using *p*-nitrophenyl-*N*-acetyl-ß-D-glucosaminide as a substrate. The assay for total hexosaminidases activity was performed according to Slawson *et al* (25) at pH 5.3. The results showed that the activity was higher in the majority of tumor samples than in

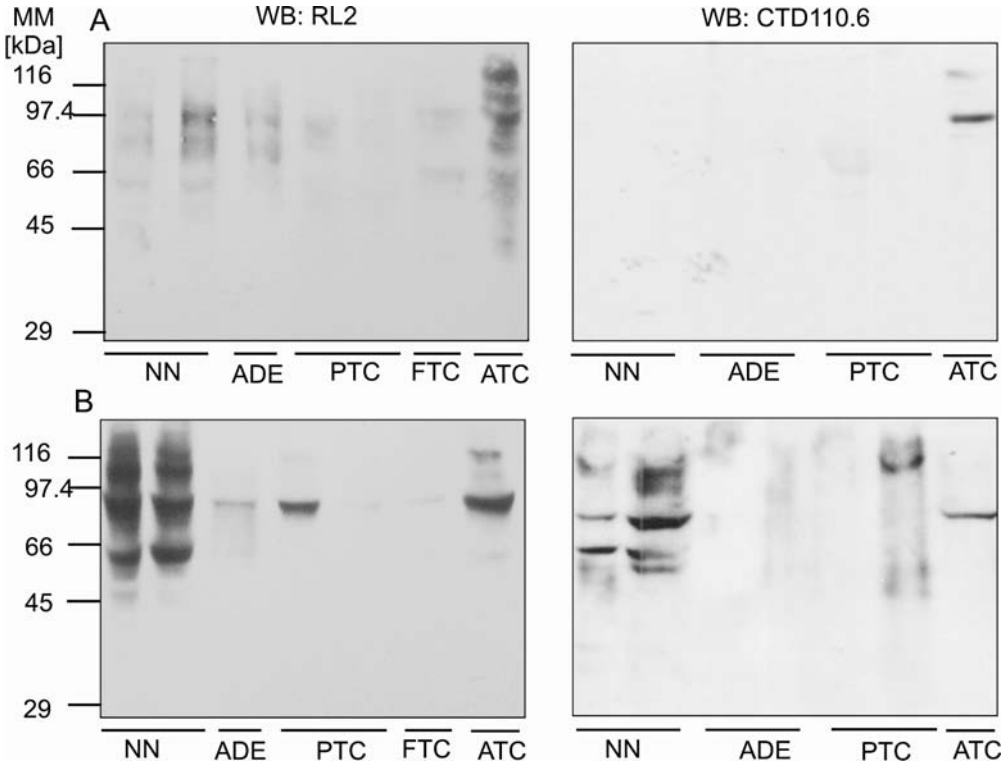

Figure 3. *O*-GlcNAc-modified proteins in thyroid pathological specimens. Cytoplasmic (A) and nuclear (B) proteins (50 μg) from non-neoplastic lesions (NN), follicular adenomas (ADE), papillary carcinomas (PTC), follicular carcinomas (FTC) and anaplastic carcinomas (ATC) electrophoresed on 8% acrylamide slab gels were transferred onto Immobilon-P membranes and tested with CTD 110.6 and RL2 antibodies. Molecular mass standard is shown on the left.

non-neoplastic lesions (Fig. 1). Acidic hexosaminidase activity ranged from 3.1 to 15.8 nmoles/min/mg in non-neoplastic lesions, from 5.6 to 22.5 in adenomas, from 6.5 to 37.5 in papillary carcinomas, from 17.2 to 36.1 in follicular carcinomas and from 23.9 to 40.5 in anaplastic carcinomas.

The fact that lysosomal hexosaminidase and *O*-GlcNAcase respond differently to inhibitors was a key factor that allowed us to differentiate *O*-GlcNAcase activity from the total hexosaminidase activity. *O*-GlcNAcase activity was determined at pH 6.45 and two different methodological approaches were used. In the first case we applied the method of Slawson *et al* (25). Neutral hexosaminidase activity (pH 6.45) was estimated in the presence or absence of *O*-GlcNAcase inhibitor-streptozotocin (STZ). *O*-GlcNAcase activity was estimated by the difference from the total neutral hexosaminidase activity and STZ inhibited activity. In the second case hexosaminidase activity was measured in the presence of GalNAc, a widely used inhibitor of acidic ß-hexosaminidase. GalNAc does not inhibit *O*-GlcNacase. The results of *O*-GlcNAcase activity estimated by both methods in different thyroid lesions are shown in Fig. 2. *O*-GlcNAcase activity is increased in the majority of cancer samples in comparison with benign lesions i.e. non-neoplastic lesions and adenomas. The mean *O*-GlcNAcase activity obtained with both methods was 0.703 nmol/min/mg in the case of non-neoplastic lesions, 0.709 in adenoma, 1.277 in papillary carcinomas, 1.172 in follicular carcinomas and 1.575 in anaplastic carcinomas.

O-GlcNAc-modified proteins level was also analyzed in thyroid lesions. Protein samples of cytoplasmic and nuclear fractions separated by electrophoresis in 8% slab polyacrylamide gels were transferred onto Immobilon-P transfer membranes. Equal protein loading was evidenced by Ponceau S staining of transferred proteins (data not shown). Representative patterns of cytoplasmic and nuclear glycoproteins binding CTD 110.6 or RL2 antibody from thyroid lesions are shown in Fig. 3. *O*-GlcNAc-modified proteins in thyroid cells have a predominantly nuclear distribution and their molecular masses ranged from 52 to 116 kDa. Nuclear fraction of tumor samples were less abundant in *O*-GlcNAc than non-neoplastic lesions. However, glycoprotein with molecular mass of ~74/76 kDa was even more abundant in some tumor samples than in adenoma or non-neoplastic samples.

Discussion

Herein, we analyzed the activity of acidic, lysosomal and neutral, cytosolic ß-*N*-acetyl*O*-D-hexosaminidases in human benign and malignant thyroid lesions, i.e. nodular goiters, adenomas, papillary carcinomas, follicular carcinomas and anaplastic carcinomas. Our results showing increased activity of lysosomal hexosaminidases in thyroid cancers are in agreement with most previous literature data. The enhancement of lysosomal ß-*N*-acetylglucosaminidase degradative activity in different human cancers, i.e. gastric, renal, breast, lung, glial and salivary gland is well documented (6-15).

In contrast to relatively well characterized lysosomal ß-hexosaminidase A and B isozymes little information is currently available on cytosolic ß-*N*-acetylglucosaminidase

(*O*-GlcNAcase) activity in cancers. For the first time in literature, our studies demonstrated increased activity of this enzyme in thyroid cancers in comparison to benign lesions. So far, one study has been published concerning the activity of *O*-GlcNAcase in human cancer tissues. Slawson *et al* (25) studied *O*-GlcNAcase activity and *O*-GlcNAc level in breast carcinomas and matched adjacent breast tissue. They found *O*-GlcNAcase activity to be significantly higher in tumor tissue compared to matched normal tissue. The total amount of *O*-GlcNAc monoglycosylation was significantly decreased in the tumor tissue especially of proteins in the molecular mass range of 45-65 kDa. However, the variety of modified proteins was greater in tumor tissue compared to adjacent tissue (25).

We analyzed *O*-GlcNAc level in nuclear and cytosolic fractions. Our results show that *O*-GlcNAc in thyroid cells has a predominantly nuclear distribution. It is in agreement with literature data showing that the greatest concentrations of *O*-GlcNAc modified proteins are in the nuclear pore complex and in chromatin. Many transcription factors as well as RNA polymerase II are also *O*-GlcNAcylated. It is suggested that *O*-GlcNAc plays a role in nuclear localization of some proteins by acting as an NLS (nuclear localization signal) or as a nuclear retention signal. Irrespective of the cause, the fact remains that the nuclear forms of proteins such as c-Myc, Tau, Stat5a, Pax-6, ELF-1, Sp1 and mTOR α4 phosphoprotein are proportionally more *O*-GlcNAc modified than cytoplasmic forms for these proteins (20).

We found that in most tumor samples *O*-glycosylation was lower than in non-neoplastic lesions. However, in several cases glycoprotein with molecular mass 74/8 kDa was more abundant than in non-neoplastic or adenoma samples. We expected that higher *O*-GlcNAase activity would be correlated with a lower *O*-GlcNAc level and it was true in most cases but not in all. It should be remembered that *O*-GlcNAc level depends not only on *O*-GlcNAcase activity but also on *O*-GlcNAc transferase. In the future it would also be necessary to analyze the activity of *O*-GlcNAc transferase. It is probable that modification of specific proteins is more important for the behavior of cancer cells than total *O*-GlcNAc level. *O*-GlcNAcase can be involved in the selective removal of *O*-GlcNAc on certain cellular proteins.

At present, the function of *O*-GlcNAc modification is studied thoroughly. *O*-GlcNAc is an inducible and dynamically cycling post-translational modification that can regulate many cellular processes (26-28). There are data showing that changes of *O*-GlcNAc level caused by PUGNAc treatment in different cell types can affect phosphorylation of proteins involved, for instance, in PI3K/Akt signaling pathway, cellular stress response, cell cycle, transcription and protein degradation (27,28). The involvement of *O*-GlcNAcylation in the pathogenesis of the diseases such as diabetes and Alzheimer's disease is strongly suggested but the role of *O*-GlcNAcylation in tumorogenesis and progression of cancer is still under-investigated. It is probable that changes in *O*-GlcNAc modification level in cancer cells may significantly influence their behavior i.e. proliferation activity and/or metastatic potential. Understanding the relationship between aberrant *O*-GlcNAcylation and cancer behavior may be beneficial for cancer prevention and treatment.

Acknowledgements

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