

Measuring O-GlcNAc cleavage by OGA and cell lysates on a peptide microarray



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ABSTRACT

O-GlcNAcylation is a post-translational modification resulting from the addition of an N-acetylglucosamine moiety to the hydroxyl groups of serine and threonine residues of nuclear and cytoplasmic proteins. In addition, O-GlcNAcylated proteins can be phosphorylated, which suggests the possibility for crosstalk between O-GlcNAcylation and phosphorylation. Dysregulation of O-GlcNAcylation affects cell signaling, transcriptional regulation, cell cycle control and can e.g. lead to tumorigenesis and tumor metastasis. There is a strong demand for efficient analytical techniques to better detect and investigate this abundant modification and its role in cancer. Herein we demonstrated the utility of an O-GlcNAcylated peptide array to examine O-GlcNAcase (OGA) activity and substrate specificity of both purified protein as well cell lysates of different cancer cell lines. Using this microarray, we clearly observed OGA activity and also inhibition thereof by OGA inhibitor thiamet G. Interestingly, different levels of OGA activity were observed of lysates derived from different cancer cell lines. This suggests that the tool may be useful in cancer research and biomarker development.

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Introduction

Cancer cells have a different metabolism than normal cells. Cancerous cells produce ATP by promoting a high rate of glycolysis followed by lactic acid fermentation (Warburg effect) rather than oxidative phosphorylation in mitochondria. These cells also need carbon- and nitrogen-rich nutrients for cell proliferation [1]. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), is located at the centre of the glucose, nitrogen, fatty acid and nucleic acid metabolic pathways [2]. UDP-GlcNAc is the metabolic substrate for O-GlcNAc transferase (OGT). OGT uses UDP-GlcNAc to catalyze O-GlcNAc addition to the free hydroxyl group of serine or threonine residues of cytoplasmic and nuclear proteins. This modification is subsequently removed by the glycosidase enzyme O-GlcNAcase (OGA) [3]. Dysregulation of the O-GlcNAcylation modification affects cell signaling, transcriptional regulation and cell cycle control and can lead to tumorigenesis and tumor metastasis [4–6]. Most oncogenic factors such as p53, MYC, NF- κ B, Snail, HCF-1, β -catenin and p27 are O-GlcNAcylated [6]. In addition, crosstalk between O-

GlcNAcylation and phosphorylation was shown especially for oncogenic factors [7,8]. For example, the *in vivo* stability of the tumor suppressor protein p53 is regulated by phosphorylation. Phosphorylation at Thr155 leads to proteasomal degradation of this protein, which keeps it at a low abundance. O-GlcNAcylation of Ser149 decreases Thr155 phosphorylation and stabilizes p53 [9] protein expression. In hepatocellular carcinoma, a dynamic interplay between O-GlcNAcylation and phosphorylation of tumor suppressor p27 regulates cell proliferation [10].

A recent study showed a link between fatty acid synthase (FAS) and OGT [11]. Both FAS and OGT indirectly use the same substrate, glucose, which is highly increased in cancer cells. FAS is overexpressed in breast, colon, esophageal, lung, melanoma, ovarian, pancreatic, prostate and stomach cancer [11]. An elevated O-GlcNAcylation level has been observed in aggressive lung tumor [12], colon tumor [13], and chronic lymphocytic leukemia [14]. On the other hand, the O-GlcNAc level is decreased in breast cancer [15] and thyroid cancer [16].

There is a major challenge to detect and study O-GlcNAcylation. This is due to its dynamic, substoichiometric and labile O-glycosidic linkage. The traditional methods such as the wheat germ agglutinin (WGA) lectin [17], pan-specific O-GlcNAc antibodies [18,19], or

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radiolabeling using β -1,4-galactosyltransferase (GalT), which transfers [^3H]Gal from UDP-[^3H]galactose to terminal GlcNAc groups [20] have limited sensitivity. Proteins with weakly expressed or few O-GlcNAc sites are not detected by the WGA lectin [17]. Furthermore, the WGA lectin also detects GlcNAc on both N- and O-linked GlcNAc moieties and does so with higher sensitivity. For a definitive identification of O-GlcNAc, WGA lectin must be used in conjunction with other treatments [20]. The success of β -1,4-galactosyltransferase (GalT) labeling for detection is dependent on the accessibility of O-GlcNAc residues to the transferase which is provided by denaturation of the substrate protein by SDS. In some cases, O-GlcNAc is not fully accessible to the enzyme even after SDS treatment [20]. Recently, the biotinylated GalNAz labeling using GalT^{Y289L} allowed the enrichment of the GalNAz labelled peptides. Although this approach improved the sensitivity of the detection [21], the harsh conditions required to disrupt the biotin-streptavidin interaction may hydrolyze the labile O-GlcNAc moiety [22]. In the present study, a novel and dynamic serine O-GlcNAc peptide microarray is developed which includes immobilized O-GlcNAcylated peptides on a chip. This chip is able to identify OGA activity in both purified protein as well as in cell lysates of different cancer cell lines. The chip method has the potential to simultaneously evaluate hundreds of glycopeptides, may lead to new specificity insights and the role of other cellular proteins in this process. Furthermore, it might be a promising tool for the study of cancer and biomarkers, diagnostics and personalized medicine.

Material and method

Materials

Isopropyl 1-thio- β -D-galactopyranoside (IPTG) and OGA inhibitor (3aR,5R,6S,7R,7aR)-2-(ethylamino)-3a,6,7,7a-tetrahydro-5-(hydroxymethyl)-5H-Pyrano [3,2-d]thiazole-6,7-diol (Thiamet G) were purchased from sigma Aldrich (Zwijndrecht, The Netherlands). The mouse monoclonal Anti-O-Linked N-Acetylglucosamine antibody [RL2] (ab2739) was obtained from Abcam (London, England). A FITC-conjugated goat anti-mouse secondary antibody was purchased from Thermo Scientific (Bleiswijk, Netherlands). All PamChip4 microarray chips were provided by PamGene (PamGene International, The Netherlands) and printed at their facilities.

Overexpression and purification of O-GlcNAcase

Plasmid encoding O-GlcNAcase cDNA (GenBank accession number AB014579) was kindly provided by Dr. Gerald Warren Hart from the Johns Hopkins University School of Medicine. *Escherichia coli* BL21 (DE3) transformed with the expression plasmid was grown in Luria–Bertani (LB) broth supplemented with 50 $\mu\text{g}/\text{mL}$ ampicillin at 37 °C at 200 rpm. Protein expression was initiated by the addition of IPTG (Isopropyl-L-thio- β -D-galactopyranoside) to a final concentration of 0.1 mM when the optical density at 600 nm was reached around 0.6. The culture was further grown for 10 h at 16 °C with shaking at 200 rpm. Cells were harvested by centrifugation and suspended in 50 mM phosphate buffer, pH 7.5, 300 mM NaCl, 0.1% triton, 5% glycerol and complete mini EDTA-free protease inhibitor cocktail for 10 min on ice. The lysate was then disrupted by sonication. Cell debris was removed by centrifugation (13,000 g, 30 min, 4 °C), and the supernatant was loaded onto a His60 Ni Gravity column (Clontech). After several times washing, the bound protein was eluted using 50 mM phosphate buffer pH 7.5, 300 mM NaCl, and 300 mM imidazole. The eluted fraction was concentrated using a 100 kDa molecular weight cut off Amicon unit by centrifugation at 4 °C for 10 min at 5000 rpm. Protein concentration was measured by the BCA method. The purity and apparent molecular

mass were determined by SDS-PAGE.

Synthesis of peptides and O-GlcNAcylated peptides

Synthesis of all peptides including the O-GlcNAcylated peptides (Fig. 1A) was achieved by following a standard Fmoc SPPS strategy on a Symphony Multiple Peptide Synthesizer starting from a Rink amide resin. The Fmoc amino acids and a Fmoc-Ser(β -D-GlcNAc(Ac)₃)-OH were used. Deprotection was performed using 20% piperidine in DMF, and coupling was performed using 1:0.9:2 amino acid/HBTU/DIPEA in DMF. The N-terminus of the peptides was then acetylated with Ac₂O while the O-GlcNAcylated peptides were still attached to the resin. On-Resin acetyl deprotection of GlcNAc was carried out with 80% hydrazine in methanol. The resins then were incubated with a 10 mL mixture of TFA (trifluoroacetic acid): H₂O: triisopropylsilane (TIPS): 1, 2-ethanedithiol (EDT) (9:0.5:0.25:0.25, v/v/v/v) and allowed to stir for 2 h at room temperature under a nitrogen atmosphere. The product was filtered and the resin was washed with TFA (2 mL) and DCM (4 mL). The residue was precipitated by the addition of pre-cooled diethyl ether and centrifugation. The precipitated peptides were dissolved in water, frozen, and overnight lyophilized. All products were stored at –20 °C. Crude peptide analysis was carried out by LC-MS.

Immobilization of peptides and O-GlcNAcylated peptides on PamChip® microarray

The synthesized peptides were immobilized in 3 different spotting concentrations (1.0, 0.6, 0.3 mM) (Fig. 1B) inside a porous membrane made of aluminium oxide which allows flow-through of the sample and thereby enhances assay sensitivity. Four arrays are combined into a PamChip 4 and were measured simultaneously. The reaction mixture was pumped up and down through the array, giving the OGA maximal opportunity to hydrolyze the O-GlcNAc from the O-GlcNAcylated peptides on each array. When the solution is temporarily placed underneath the array, the CCD camera in the workstation takes an image of each array. Functional readout is based on statistical analysis of disappearing O-GlcNAc signal on the array using fluorescently labelled anti-GlcNAc-antibody. Image analysis was performed using BioNavigator 6 software (PamGene International, The Netherlands). Each image was quantified by automated array grid finding and subsequent quantification of the signal (after local background subtraction) for each individual spot.

Cell cultures and cell lysis

The human breast carcinoma MCF7 cell line (ATCC HTB22), human HT29 (ATCC® HTB-38™) colon cancer cells, heterogeneous human epithelial colorectal adenocarcinoma Caco-2 (ATCC HTB 37) cells and fibroblasts WT ATCC were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10%(v/v) fetal calf serum (FCS) (Lonza) and 10 $\mu\text{g}/\text{mL}$ streptomycin at 37 °C in a humidified atmosphere enriched with 5% CO₂. Cells were allowed to reach a confluency of about 75%. Cells were lysed on ice for 15 min using RIPA lysis buffer with protease inhibitor (complete mini EDTA-free protease inhibitor). The cell lysate was centrifuged at 4 °C for 15 min at 15,000 \times g and the supernatant was collected. The BCA Protein Assay kit was used to determine the total protein concentration. The supernatant was then aliquoted and kept at –20 °C.

Preparation of the primary/secondary antibody mixture

A 1:1 fresh mixture of the mouse monoclonal anti-O-GlcNAc antibody (RL2) and a FITC-conjugated goat anti-mouse secondary

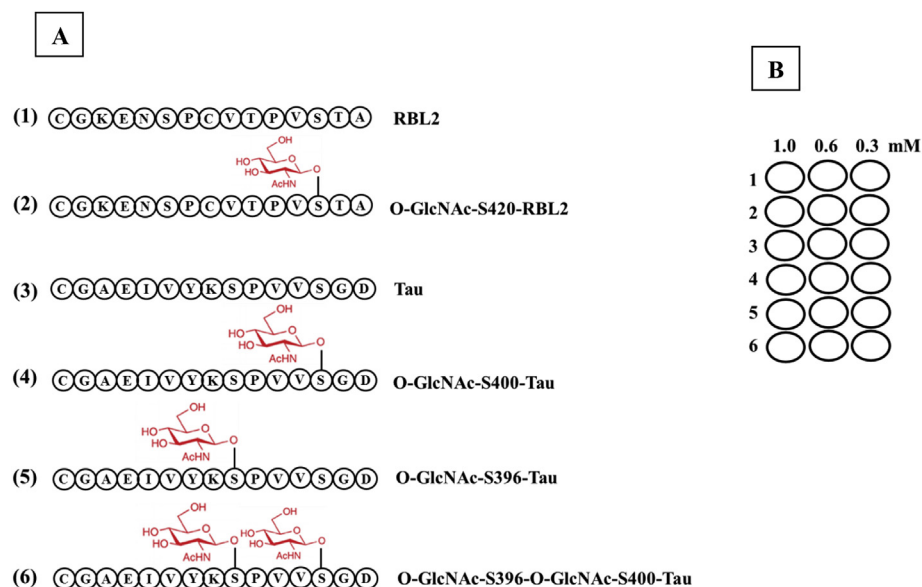


Fig. 1. A) The peptides and O-GlcNAcylated peptides immobilized on microarray. Peptides 1 and 2 are derived from the RBL2 protein. Peptides 3,4,5 and 6 are derived from the Alzheimer-linked Tau protein. B) chip layout, the synthesized peptides were immobilized in 3 different spotting concentrations 1.0, 0.6, 0.3 mM. The numbers correspond to the peptides and their location on the microarray chip.

antibody was prepared and pre-incubated at room temperature (RT) for at least 30 min before use.

OGA activity assay

OGA activity was measured in two ways. The first involved the use of *o*-chloro-*p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucosaminide (oClpNP- β -GlcNAc) as a substrate similar to reported procedures [23]. A standard curve was obtained using different concentrations of *o*-chloro-*p*-nitrophenol. The color that developed was read at 410 nm using a microplate reader (uQuant, Biotek, Instruments Inc.). The reaction mixtures (Test and Blank) in an eppendorf tube contained 100 mM citrate buffer with 200 mM sodium chloride, 0.02% Albumin (pH 5.0) and 10 mM oClpNP- β -GlcNAc incubated at 30 °C for 30 min. Then, OGA was added to the Test eppendorf tube and was incubated for exactly 10 min at 30 °C. The reactions were stopped by the addition of 200 mM borate buffer at pH 9.8. The absorbance (410 nm) of the reactions was recorded. The specific enzyme activity (Units/mL) of OGA was calculated.

The second method to measure OGA activity used the PamChip microarray. Before the addition of OGA or a cell lysate to the array, the microarray was blocked with blocking buffer (1.0% BSA in TBS). After 30 cycles (2 cycles/min) blocking, the blocking buffer was removed. The microarray was then incubated with 40 μ L of the enzyme reaction containing OGA or cell lysate (50 μ g/mL) in Britton-Robinson buffer (40 mM boric acid, 40 mM phosphoric acid and 40 mM acetic acid, pH 6.5) with or without OGA inhibitor (thiamet G, 50 μ M) for 420 cycles (1cycle/min). After washing and aspirating, antibody mixture was added to the array and was incubated for 120 cycles (2 cycles/min). A tiff image was obtained by CCD camera. Changes in O-GlcNAcylated peptides were detected by the fluorescent signal intensity which was produced by FITC conjugated secondary antibody in antibody mixture while the anti-O-GlcNAc antibody bound to the O-GlcNAc moiety of peptides.

Statistical analysis

All experiments were repeated 3 times. The strongest signal

intensity of the control array extracted from the BioNavigator 6 software referred as 100% signal intensity. The percentage of the signal intensities of O-GlcNAcylated peptides in the other array calculated according to this reference signal intensity. The average of these percentages was used. The low activity of OGA enzyme resulted in a strong signal intensity. The error bar indicates the SEM (N = 3).

Results

O-GlcNAcylated peptides were recognized by FITC conjugated anti-O-GlcNAc-antibody

To investigate whether the O-GlcNAcylated peptides are recognized by anti-O-GlcNAc-antibody, the antibody mixture (mixture of mouse monoclonal Anti-O-Linked N-Acetylglucosamine antibody (RL2) and FITC conjugated secondary antibody, 1:1) were added to one of arrays (Fig. 2).

As controls, to the first array only OGA buffer, to the second array only primary antibody and to the third array only secondary antibody was added. The three first arrays showed no fluorescence signal. In the fourth array, the peptides without O-GlcNAc moiety (RBL2 and Tau) showed no signal. The signal intensity of each O-GlcNAcylated peptide was clear and unique. The intensity of the signals derived from the O-GlcNAcylated peptide was dependent on the spotting concentration of the peptide. The highest signal in each row was due to the peptide with the highest spotting concentration (1 mM). Peptide O-GlcNAc-S420-RBL2 showed the highest signal intensity. A weaker signal was observed for O-GlcNAc-S396-Tau (Fig. 2) even at the highest concentration of the peptide. The signal intensity of the peptide O-GlcNAc-S400-Tau was higher than the peptide O-GlcNAc-S396-Tau. The peptide O-GlcNAc-S396-O-GlcNAc-S400-Tau with two O-GlcNAc moiety showed a higher signal intensity compare to that of peptides O-GlcNAc-S396-Tau and O-GlcNAc-S400-Tau but less intense than O-GlcNAc-S420-RBL2 which has only one O-GlcNAc moiety. The peptide with 0.3 mM spotting concentration showed the lowest signal intensity compared to those with 0.6 and 1.0 mM (Fig. 2).

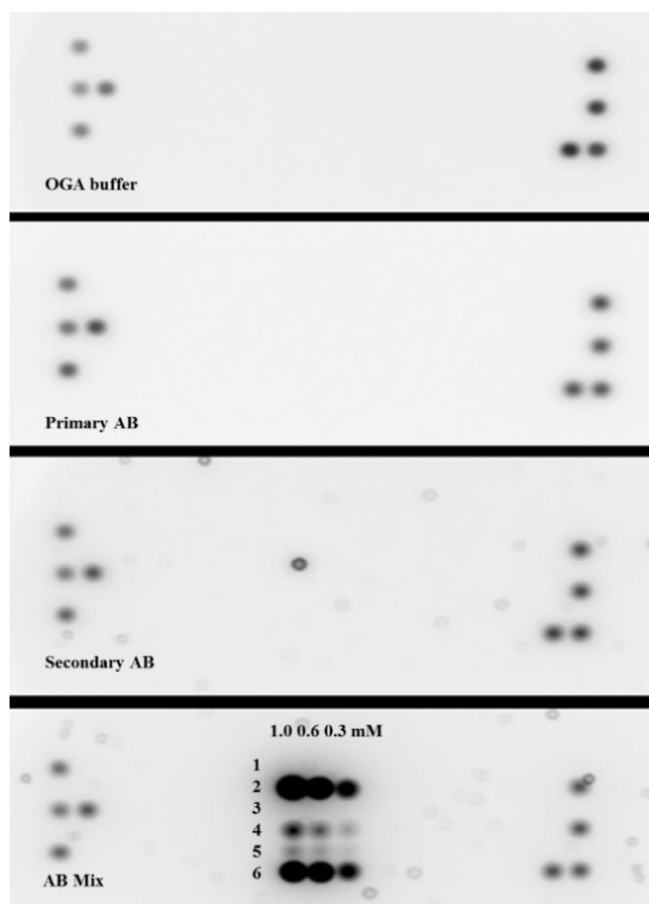


Fig. 2. Recognition of O-GlcNAcylated peptides by 1:1 mixture of anti-O-GlcNAc (RL2) and the FITC conjugated secondary antibody. The peptides 1 to 6 were immobilized on array at 3 different spotting concentrations. To the first array only OGA buffer, to the second array only primary antibody and to the third array only secondary antibody was added.

OGA can remove O-GlcNAc from the immobilized O-GlcNAcylated peptides

To investigate whether OGA can recognize O-GlcNAcylated peptides and remove O-GlcNAc from them, OGA was incubated on the microarray (Fig. 3A). A clear decrease in signal intensity was observed which was detected by the antibody mixture. The signal intensity was decreasing at all 3 different peptide spotting concentrations. In this assay, the observed decrease in signal intensity was directly proportional to enzyme activity. A low activity of OGA enzyme led to a strong signal intensity. The signal intensity was reduced to 16% of the control (no enzyme) value using the OGA enzyme.

Cell lysates show different OGA activity among the immobilized O-GlcNAcylated peptides

To investigate how OGA enzymatic activity in cell lysates would react with the immobilized O-GlcNAcylated peptides, 15 μ g of these cell lysates (Fibroblast, MCF7, HT29, Caco2) were incubated on the microarray. The fibroblast cell lysate exhibited a low OGA activity (Fig. 4A). The signal intensity of the fibroblast lysate was about 75% of the control value (Fig. 4C). The OGA activity of the Caco2 cell lysate was significantly higher than that of the fibroblast cell lysate (Fig. 4C).

The MCF7 and HT29 cell lysates (Fig. 4B) showed the most OGA activity. A remaining signal in these cell lysates was only seen for the O-GlcNAc-S420-RBL2 peptide. The O-GlcNAc moiety was removed from most of the O-GlcNAcylated peptides. All three printing concentrations of the peptides showed the loss of O-GlcNAc. The percentage fluorescence intensity (Fig. 4C) is the average of the signal intensities of the O-GlcNAcylated peptides on the array compared to the strongest signal intensity from the control data. The strongest signal intensity from the control array referred to 100% signal intensity. The remaining signal intensity of the experiment with the MCF7 cell lysate was only 0.06% of the control experiment.

OGA inhibitor decreases OGA activity both in purified enzyme and also in cell lysates

Subsequently, the effect of OGA inhibitor thiamet G was studied with respect to the deGlcNAcylation activity of the MCF7 lysate. Inhibition of the OGA enzyme with 50 μ M of OGA inhibitor (thiamet G) led to higher fluorescence signals of peptides 4 and 5 (Fig. 5A). It is clear that the inhibition of OGA by thiamet G can be observed and led to an increase in the remaining O-GlcNAc signal intensity. The overall signal intensity was increased from 16% in –TG to 31.5% in +TG (Fig. 5B).

The addition of thiamet G to the MCF7 cell lysate resulted in the inhibition of OGA activity of the MCF7 cell lysate too (Fig. 5C). The signal intensity derived from the O-GlcNAc-S420-RBL2 peptide (row 2) was evaluated. The overall signal intensity was increased about 98.3% from 0.06% in –TG to 5.9% in +TG (Fig. 5D). The average of the percentages of fluorescence intensities of the O-GlcNAcylated peptides were used to calculate the percentage of signal intensity after addition of Thiamet G (Fig. 5B and D).

Discussion

Increasing number of reports indicate the central involvement of O-GlcNAcylation in tumorigenesis, which makes its detection a potentially new approach for cancer diagnosis [24–26]. An earlier study in our group using a dynamic peptide microarray demonstrated that this technology is a very useful tool for studying OGT and O-GlcNAcylation activities [27]. We are the first to design an O-GlcNAcylated peptide microarray. This microarray consists of immobilized O-GlcNAcylated peptides. Three of these peptides are derived from the Alzheimer-linked Tau protein [28] and one from the RBL2 protein. Our group and others showed that Ser 420 in RBL2 is one of the possible O-GlcNAc sites of RBL2 [27,29]. Serine 400 in Tau is a known O-GlcNAcylation site [30–32]. In the present study, we used the O-GlcNAcylated peptide microarray to study OGA activity and alteration of O-GlcNAcylation by different cancer cell lines. Our studies showed that different cancer cell lines have different O-GlcNAcase activity with MCF7 being the highest. Caco-2 and fibroblast cell lines show low OGA activity compare to HT29 and MCF7.

Earlier studies showed that O-GlcNAcase activity is higher in solid tumors from breast cancer patients, especially in more aggressive tumors [33]. By contrast, OGT protein expression showed an increase in tumor cell lines that mimic metastatic tumors [34]. Our results showed that MCF7 cells have a high O-GlcNAcase activity. Furthermore, the inhibition of OGA by the OGA inhibitor Thiamet G, was clearly visible.

Several studies showed an alteration of O-GlcNAcylation is associated with colon cancer formation and progression. Mi et al. showed that an OGA inhibitor (Thiamet G) could markedly elevate the O-GlcNAcylation of HT29 cells [12]. Using this peptide microarray, we observed an elevation in O-GlcNAcase activity in

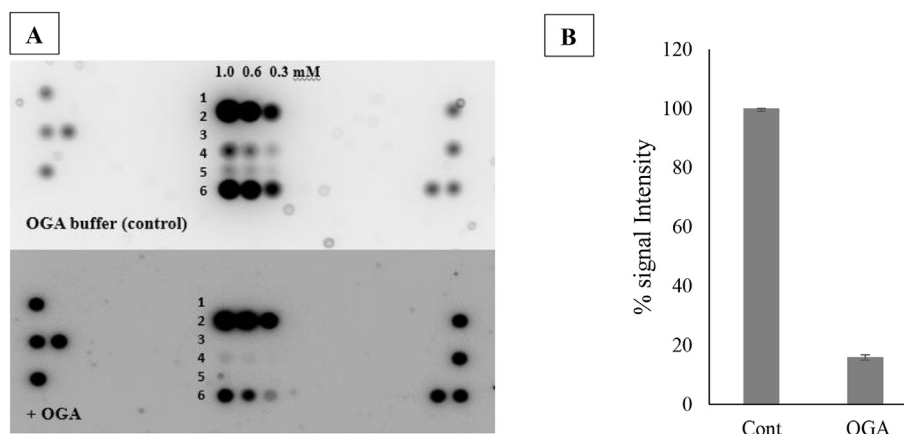


Fig. 3. OGA can remove O-GlcNAc from the immobilized O-GlcNAcylated peptides. A) OGA buffer was added to the first array and 15 μ g of pure OGA was added to the second array. After the incubation time, the array was washed and the antibody mixture (1:1 primary antibody and secondary antibody) was added to the arrays. B) The strongest signal intensity of the control array extracted from the BioNavigator 6 software referred as 100% signal intensity. The percentage of the signal intensities of O-GlcNAcylated peptides in the other array calculated according to this reference signal intensity. The average of these percentages was used. Error bar indicate the SEM (N = 3).

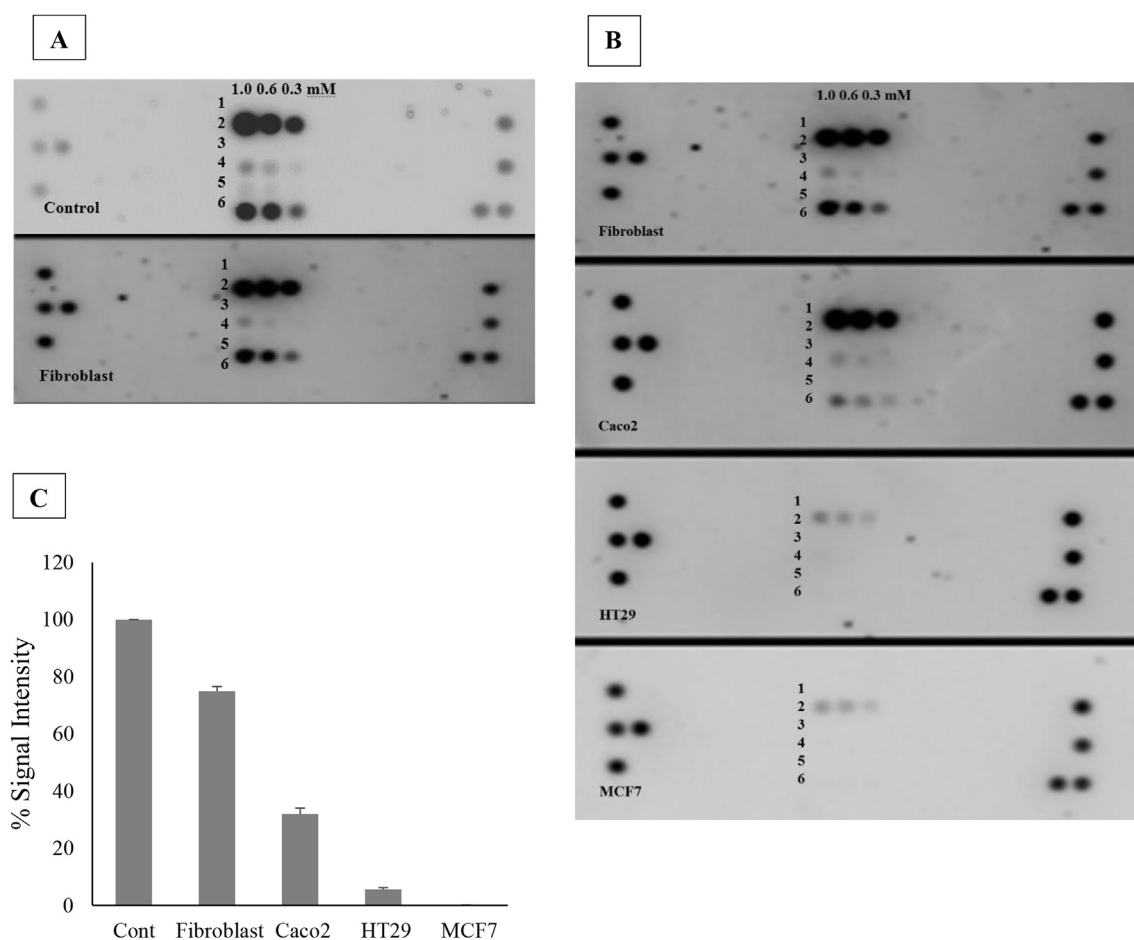


Fig. 4. A) 15 μ g of fibroblast cell lysate was added to the peptide microarray. As a control, to the first array only OGA buffer was added. B) 15 μ g of each cell lysates fibroblast, Caco-2, HT29, MCF7 was added to each array. After the incubation time, the array was washed and aspirated and antibody mixture was added to the array. C) The strongest signal intensity of the control array extracted from the BioNavigator 6 software referred as 100% signal intensity. The percentage of the signal intensities of O-GlcNAcylated peptides in the other arrays calculated according to this reference signal intensity. The average of these percentages was used. Error bars indicate the SEM (N = 3).

HT29 cells. The present results encourage further investigation, improvements and validation of this array. An advanced model of this microarray could be used as a tool in cancer studies and

possibly even in diagnostics and the prediction of therapeutic responses.

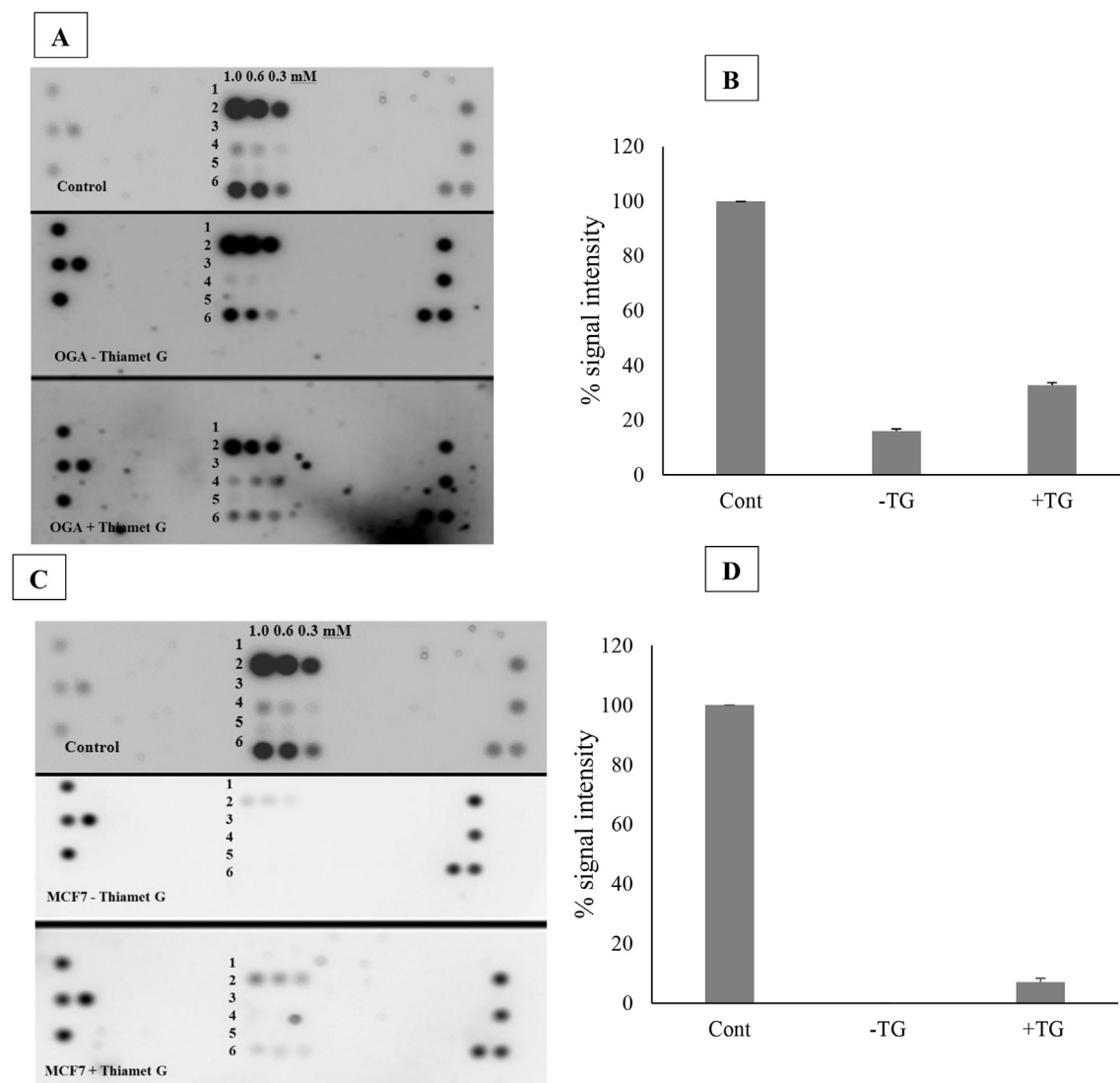


Fig. 5. (A) 15 μ g OGA was preincubated with and without Thiamet G (50 μ M) for 30 min on ice and added to array. After incubation time, the array was washed and aspirated and antibody mix was added to the array. (B) The strongest signal intensity of the control array extracted from the BioNavigator 6 software referred as 100% signal intensity. The percentage of the signal intensities of O-GlcNAcylated peptides in the other array calculated according to this reference signal intensity. The average of these percentages was used. Error bar indicate the SEM (N = 3). (C) 15 μ g MCF7 cell lysate was preincubated with and without Thiamet G (50 μ M) for 30 min on ice and added to array. After incubation time, the array was washed and aspirated and antibody mix was added to the array. (D) Same as B. Error bar indicate the SEM (N = 3).

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