Comprehensive Profiling of N-Linked Glycosylation Sites in HeLa Cells Using Hydrazide Enrichment

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ABSTRACT: The adenocarcinoma cell line HeLa serves as a model system for cancer research in general and cervical cancer in particular. In this study, hydrazide enrichment in combination with state-of-the-art nanoLC–MS/MS analysis was used to profile N-linked glycosites in HeLa cells. N-Linked glycoproteins were selectively enriched in HeLa cells by the hydrazide capture method, which isolates all glycoproteins independent of their glycans. Nonglycosylated proteins were removed by extensive washing. N-Linked glycoproteins were identified with the specific NXT/S motif and deamidated asparagine (N). Deglycosylation was carried out in both H₂¹⁶O and H₂¹⁸O to confirm the deamidation. NanoLC–MS/MS analysis indicated that the method selectively enriched at least 100 fold N-linked glycosites in HeLa cells. When both the membrane and cytosolic fractions were used, a total of 268 unique N-glycosylation sites were identified corresponding to 106 glycoproteins. Bioinformatic analysis revealed that most of the glycoproteins identified are known to have an impact on cancer and have been proposed as biomarkers.

KEYWORDS: N-linked glycoproteins, hydrazide capture, HeLa cells, cancer, biomarkers

1. INTRODUCTION

Since its introduction as the first human cell line, HeLa has served as a model system for cancer research in general and cervical cancer in particular. In addition, research on HeLa cells was pivotal in our understanding of many biological processes.¹ Originally, HeLa was derived from a cervical adenocarcinoma, caused by human papillomavirus.² After breast cancer, cervical cancers represent the second most common cancer in women.

Current cancer treatment options include surgery, radiotherapy, chemotherapy, or combinations thereof. Biomarker research is needed for early detection, effective prevention, and treatment of cancer. It has, for instance, been shown that radiotherapy resistance can be diagnosed with biomarkers.³ However, the discovery of biomarkers is challenging and requires sensitive and selective methods that can determine low abundance proteins even in complex samples. Additionally, the sample amount and number of cells may also be limited making a selective sample preparation necessary in combination with state-of-the-art nanoLC–MS/MS. It has been recognized that the onset of tumors and metastases involve N-glycosylated proteins.^{4–6} In fact, glycoproteins are implicated in diverse biological functions that are essential for tumor development, growth, and metastases, including cell–matrix and cell–cell recognition, enzymatic activity, intracellular transport, protein folding, and regulation.⁷ Consequently, variations in glyco-

proteins are associated with diseases and may therefore function as biomarkers of great clinical relevance.^{8,9}

Glycoproteins can be categorized in mainly two groups: N-linked and O-linked proteins. N-Linked glycoproteins, where the glycan is linked to asparagine (N), are identified by the NXS/T motif where X can be any amino acid except proline (P). In the O-linked glycoproteins, the glycans are attached to either serine or threonine (S/T). Cell-surface or secreted glycoproteins, likely biomarkers, are predominantly N-linked.¹⁰

There are different strategies to isolate and enrich glycoproteins, and the most common are lectin based,^{11–15} boronate affinity chromatography,^{16–18} HILIC,^{19,20} and the hydrazide capture method.^{21–26} Lectins interact preferentially with certain glycans, and it is therefore necessary to use a combination of several lectins to isolate the majority of glycoproteins. Boronate affinity chromatography, HILIC, and the hydrazide capture method are universal methods and can isolate glycoproteins independent of the glycan structure. The hydrazide capture method has recently been applied to isolate both glycoproteins and glycopeptides in different cell lines and bacteria,^{27–31} saliva,³² and rat myocardium³³ and to explore the effect of oxidants on oxidative stress in plasma in diabetic rats.³⁴

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Here, we use the hydrazide capture method to selectively enrich N-linked glycosites in HeLa cells. The method was applied for both membrane and cytosolic proteins. The N-linked glycoproteins were identified by the characteristic NXS/T motif and deamidated asparagine. More than 250 unique glycosylation sites were identified including novel glycosylation sites in known and novel glycoproteins. Interestingly, when compared to the membrane fraction more glycosylation sites were identified in the cytosol fraction. Lastly, most of the N-linked glycoproteins identified are known to have an impact on cancer.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% Pen/Strep, and PBS was purchased from Invitrogen (Carlsbad, CA). Water, acetonitrile (ACN) (Chromasolv LC-MS quality), trifluoroacetic acid (99+%), formic acid (99%), ammonium bicarbonate (ABC), DL-dithiothreitol (DTT), HEPES, iodoacetamide (IAM), octyl- β -D-1-glucopyranoside, sodium acetate (NaOAc), sodium metaperiodate (NaIO₄), urea and triethylammonium bicarbonate were supplied by Sigma-Aldrich (St. Louis, MO). Methanol (MeOH) was from J.T. Baker by Avantor Performance Materials (Center Valley, PA).

Sodium chloride (NaCl) was from Mallinckrodt Chemicals (Philipsburg, NJ) and Tris-HCl from MP Biomedicals, Inc. (Illkirch, France). H₂¹⁸O (95–98 atom % ¹⁸O) was from Isotech (Miamisburg, OH). Sequencing-grade modified porcine trypsin was purchased from Promega Corp. (Madison, WI) and PNGase F and N-glycanase from Prozyme (Hayward, CA). Complete mini EDTA-free protease inhibitor was supplied by Roche Diagnostics GmbH (Mannheim, Germany). UltraLink hydrazide resins were supplied by Pierce, Thermo Scientific (San Jose, CA).

2.2. Cell Culture and Oxidation

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% Pen/Strep, and 2.5% HEPES. Cells were grown to 70% confluency in T-150 flasks (Becton Dickinson Labware, Franklin Lakes, NJ) before the media was removed and the cells ((55–80) × 10E6) were washed with 1 × PBS three times. The cells were harvested by centrifugation (1200 g, 5 min, 4 °C) and subsequently oxidized with 10 mM sodium metaperiodate for 1 h in the dark at room temperature with gentle end-over-end mixing. A mixture of 100 mM sodium acetate and 150 mM sodium chloride (pH 5.5) was used as oxidation buffer. The cell pellet was washed three times with 10 mL 1 × PBS to remove excess NaIO₄.

2.3. Cell Lysis and Membrane Preparation

The cell pellet was resuspended in 2 mL of lysis buffer (1% solution (20 mM Tris-HCl, 150 mM NaCl, pH 7.5), 20 mg of the nonionic detergent octyl- β -D-1-glucopyranoside, and one tablet of complete mini EDTA-free protease inhibitor. Cells were homogenized using an ultrasound probe on ice and subsequently centrifuged at 38000g, 1.5 h, 4 °C (Optima L-90K Ultracentrifuge, Beckman Coulter, Inc., Brea, CA) to isolate the membrane proteins. Prior to the hydrazide capture, the membrane pellet was redissolved in 600 μ L of a mixture of 1% solution (150 mM NaCl, pH 7.5), 20 mg of the nonionic detergent octyl- β -D-1-glucopyranoside, and one tablet of

complete mini EDTA-free protease inhibitor. An ultrasound probe was used to dissolve the membrane pellet completely. Cytosolic proteins were stored at –20 °C and thawed prior to hydrazide capture.

2.4. Hydrazide Capture of Membrane Proteins

Oxidized membrane proteins (600 μ L) were mixed with 2.4 mL of hydrazide resins (1_{protein}/4_{hydrazide} ratio, v/v) and incubated at room temperature for 40 h with end-over-end mixing. The hydrazide resins were washed twice with 50 mM ABC prior to the mixing. Proteins not retained on the resins were removed by centrifugation (500g, 5 min) and subsequently washed three times with an equal volume of 8 M urea and 100 mM ABC, pH 8.4 and processed as described in the Supporting Information. The hydrazide resins were transferred to a 30 K Amicon Ultra centrifugal filter (15 mL, Millipore, Billerica, MA). Deionized water (1.5 mL) was added to the resins to increase the volume. The proteins were reduced with 50 mM DTT incubated for 1 h and alkylated with 500 mM iodoacetamide IAM for 30 min. Both reduction and alkylation were carried out with gentle end-over-end mixing at room temperature in the dark. The filter was centrifuged to remove the excess of DTT and IAM and washed three times with 150 mM NaCl and 50 mM ABC, respectively. Proteins were dissolved in 1 mL of 50 mM ABC and digested overnight at room temperature using 20 μ g of sequencing grade modified porcine trypsin. Nonglycosylated peptides were recovered by centrifugation (nonglycopeptide). The hydrazide resins were subsequently washed with 2 × 1 mL of 1.5 M NaCl, 2 × 1 mL of ACN/0.1% TFA (80/20, v/v), 2 × 1 mL of MeOH, 2 × 1 mL of water, and 2 × 1 mL of 8 M urea/100 mM ABC (pH 8.4). The resulting 10 wash fractions were combined prior to the sample cleanup. The nonglycopeptide and wash fractions were analyzed by LC–MS/MS and combined in the subsequent analysis. A set of control samples was prepared by the same procedure except oxidation with NaIO₄ was eliminated.

2.5. Hydrazide Capture of Cytosolic Proteins

The cytosolic proteins were oxidized by adding 10 mM NaIO₄ in 0.5 mL of oxidation buffer (100 mM NaAc and 150 mM NaCl, pH 5.5) and incubated for 1 h at room temperature with end-over-end rotation. The sample was transferred to a 3K Amicon centrifugal filter (0.5 mL, Millipore) and washed with 0.5 mL of PBS three times to remove excess NaIO₄. A 100 μ L portion of a mixture of 1% solution (150 mM NaCl, pH 7.5), 20 mg of octyl- β -D-1-glucopyranoside, and one tablet of complete mini EDTA-free protease inhibitor were added to the proteins. A 400 μ L portion of ABC prewashed hydrazide resins (1_{protein}/4_{hydrazide} ratio, v/v) was added, and the sample was conjugated to the resins at room temperature with end-over-end mixing overnight. The hydrazide resins were transferred to an Eppendorf tube and centrifuged and washed three times with 8 M urea in 100 mM ABC (pH 8.4) to recover the proteins not bound to the resin (flow-through and wash).

Hydrazide resins were transferred to a 30 K Amicon centrifugal filter, and 400 μ L of H₂O was added to increase the total volume. The proteins were reduced with 50 mM DTT (incubated for 1 h) and alkylated with 500 mM IAM (30 min). The reduction and alkylation was carried out with gentle end-over-end mixing at room temperature in the dark. The excess of DTT and IAM was removed by centrifugation, and the proteins were washed three times with 150 mM NaCl and 50 mM ABC, respectively. The proteins were dissolved in 350 μ L of 50 mM ABC and digested overnight at room

temperature using 20 μg of sequencing grade modified porcine trypsin. The conditions for recovering the nonglycosylated peptides were the same as for membrane proteins described above. A set of control samples was prepared by the same procedure as described for the membrane proteins.

2.6. Deglycosylation and Release of N-Linked Glycopeptides

The N-linked glycopeptides were released from the hydrazide resins by incubating 100 μL of washed resins with 500 μL of 50 mM ABC and either 4 μL of PNGase F or 2 μL of N-glycanase overnight at 37 $^{\circ}\text{C}$. For deglycosylation in H_2^{18}O , 200 μL of 50 mM ABC and 100 μL of hydrazide resins were evaporated to remove all traces of H_2^{16}O . To the vial with dried ABC were added 75 μL of H_2^{18}O and PNGase F or N-glycanase, not dried, and the mixture was mixed well before being transferred to the dried hydrazide resins and incubated at 37 $^{\circ}\text{C}$ overnight.

2.7. Protein Digestion in Unenriched Samples

Approximately 50 μL of membrane proteins and cytosolic (without hydrazide capture) were lyophilized and redissolved in 50 μL of 8 M urea/100 mM Tris-HCl (pH 8.5). The proteins were reduced and alkylated with 50 mM DTT and 500 mM IAM as described above. A 100 μL portion of 100 mM Tris-HCl (pH 8.5) was added to the reduced and alkylated samples to decrease the concentration of urea to 1.4 M. Sequencing grade modified porcine trypsin (20 μg) was dissolved in 200 μL of water and added to the samples. Protein digestion was carried out at room temperature overnight with end-over-end mixing. The enzymatic protein digestion was quenched by addition of 10 μL of 50% formic acid.

2.8. Sample Cleanup

All samples were lyophilized and resuspended in 0.1% TFA. The samples were desalted using the Sep-Pak 3 cm^3 (200 mg) tC18 cartridge (Waters Corporation, Milford, MA) and if needed in-house made StageTips (Empore High Performance Extraction disks (C18) 3 M Center, St. Paul, MN).^{35,36} The Sep-Pak 3 cm^3 tC18 SPE was conditioned with 2 $\times$ 1 mL of MeOH, 2 $\times$ 1 mL of ACN/0.1% TFA (80/20, v/v), and 3 $\times$ 1 mL of 0.1% TFA. The sample was loaded on the Sep-Pak in 1 mL 0.1% TFA and washed with 0.1% TFA. The peptides were eluted in 3 $\times$ 0.5 mL of ACN/0.1% TFA (80/20, v/v). The C18 StageTips were activated by 50 μL of MeOH, 50 μL of ACN/0.1% TFA (80/20, v/v), and 2 $\times$ 50 μL of 0.1% TFA. The sample was subsequently loaded in 50 μL of 0.1% TFA prior to washing with 0.1% TFA before the peptides were eluted with 3 $\times$ 50 μL . The eluates were lyophilized and dissolved in 10 μL of 0.2% formic acid.

2.9. LC-MS/MS

The nanoLC-MS/MS analyses were carried out on an LTQ Orbitrap (Thermo Fisher Scientific, Waltham, MA) with a nanospray interface coupled to an EASY-nLC pump (Proxeon, now Thermo Fisher Scientific). A 5 μL sample was loaded on a self-packed 5 μm Reprosil C18AQ (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) column (75 μm $\times$ 150 mm). Mobile phase A consisted of ACN/FA/ H_2O (2/0.2/98, v/v/v), and mobile phase B was ACN/FA/ H_2O (80/20/0.2, v/v/v). A 59 min gradient was used to separate the peptides. The gradient started at 0% B and increased linearly up to 40% within 50 min and was kept at 40% B for 1 min before it was increased to 100% B in 8 min. The flow rate was 350 nL/min. All samples were analyzed with one blank (water) in between.

The LTQ Orbitrap was operated in data-dependent mode with full scan MS performed in the Orbitrap and a dependent MS/MS in the LTQ. The ESI spray voltage was +2.10 kV, and the capillary temperature was 200 $^{\circ}\text{C}$. All spectra were acquired in positive mode in the range between m/z 300 and 1700 at a resolution of 100,000. Collision-induced dissociation (CID) was performed in the ion-trap (IT) with a 35% normalized collision energy at the default activation Q of 0.250 and activation time of 30 ms. Charge state screening and rejection were enabled, and only charge states of the precursor ion $\geq +2$ were accepted. The ten most intense peptide ions were isolated. Dynamic exclusion settings of 1 repeated count over 30 s and exclusion duration of 90 s. Xcalibur 2.0.7 (Thermo Scientific) was used to control both the EASY n-LC and the Orbitrap MS/MS.

2.10. Data Analysis

Mascot (v 2.2.06, Matrix Science, London, UK) was used for protein identification. Trypsin was chosen as the enzyme, and the number of missed cleavages was set to 2. The peptide charge was set to 2+ and 3+, and the peptide tolerance and MS/MS tolerance were 10 ppm and 0.5 Da, respectively. The IPI human database (v 3.54, 75429 entries) was searched along with a common contaminant database (262 entries). A decoy database constructed as described by Cox et al.³⁷ was appended to the target database. Cysteine carbamidomethylation was set as fixed modification while methionine oxidation, deamidation (NQ), deamidation: 18O (NQ) and Asn $\rightarrow$ Asp (N) were used as dynamic modifications for all database searches. Scaffold (v3.00.08, Proteome Software Inc., Portland, OR, USA) and WebLogo 3.1³⁸ were used to validate the data. Protein identifications were based on a minimum of two peptides with a peptide probability of 95% and protein probability of 99%. The estimated protein and peptide false discovery rate was estimated by Scaffold to be less than 1% based on the decoy database search results. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm.³⁹ Protein identifications were accepted if they could be established at greater than 95% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.⁴⁰ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

To identify which putative glycoproteins had been previously annotated as glycoproteins, all human proteins with the keyword "Glycoprotein [KW-0325]" were retrieved from UniProt in December 2011.⁴¹ The IPI identifiers of the putative glycoproteins were mapped to UniProt identifiers using PICR,⁴² and in-house scripts were then used to identify which putative glycoproteins were also annotated as glycoproteins in UniProt using the PICR mapping. Additionally, in-house scripts were used to determine if the putative glycosylation sites were previously annotated in UniProt with N-linked glycosylation sequence annotations. Transmembrane domains (TMDs) were predicted with the TMHMM2.0 algorithm (<http://www.cbs.dtu.dk/services/TMHMM/>). UniProt and David Bioinformatics Resources 6.7⁴³ were used to evaluate the enrichment of N-linked glycoproteins. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to evaluate the pathways of the detected glycoproteins.⁴⁴

3. RESULTS AND DISCUSSION

The aim of this study was to selectively isolate and profile N-linked glycosylation sites in HeLa cells using the hydrazide capture method. The workflow of the experiments conducted is shown in Figure 1. Vicinal diols of the sugar moieties of

Figure 1. Workflow diagram of the hydrazide capture method of N-linked glycoproteins in HeLa cells.

membrane proteins were selectively oxidized using sodium meta-periodate prior to cell lysis and isolation of membrane and cytosolic proteins by ultracentrifugation. In addition, the cytosolic fraction was oxidized after ultracentrifugation to capture potential glycosites in this fraction. Oxidized glycoproteins (and controls) were conjugated with hydrazide resins overnight, and subsequently centrifuged to remove those proteins that do not contain oxidizable sugar moieties (flow-through and wash). The captured glycoproteins were trypsin digested, while the sugar residues were still attached to the hydrazide resins, and thus only the nonglycosylated peptides of the glycoproteins were released and recovered by centrifugation and extensive washing. These fractions are referred to as nonglycopeptide and wash. Finally, the enriched tryptic N-linked glycopeptides were recovered by enzymatic deglycosylation in H_2^{16}O or H_2^{18}O (enriched glycopeptides). Control samples were prepared in the same manner excluding oxidation by sodium meta-periodate to explore potential unspecific interactions between the hydrazide resins and the HeLa proteins. The controls were deglycosylated in H_2^{16}O . In addition, a set of unenriched samples (membrane and cytosolic proteins) was included to determine the enrichment of N-linked glycoproteins of the hydrazide method (workflow not included in Figure 1). Two sample replicates were prepared for the oxidized glycoproteins (membrane and cytosolic) and control samples, respectively.

3.1. Identified Glycosylation Sites

Overall, 268 unique glycosylation sites have been identified in this study; 245 of those were identified in the cytosol and 111

in the membrane. While 88 glycosylation sites were identified in both the cytosol and membrane fraction, the majority were found in the cytosolic fraction. Table 1 lists the number of sites,

Table 1. Identification of Unique Glycosylation Sites in HeLa Glycoproteins from Membrane and Cytosolic Fractions

	unique glycosylation sites
membrane fraction	
known site of known glycoprotein	88
novel site of known glycoprotein	18
novel site of novel glycoprotein	5
cytosolic fraction	
known site of known glycoprotein	184
novel site of known glycoprotein	9
novel site of novel glycoprotein	52

subdivided in known and novel glycosylation sites of known and novel glycoproteins. All sites are specified in Tables S1 and S2 and best matching spectra of the deglycosylated peptides are shown in Supplementary Data 3 (Supporting Information).

We compared our results to a recent report from McDonald et al. identifying a total of 127 unique N-linked glycosylation sites in membrane associated HeLa cells using a combination of hydrazide capture and lectin affinity.⁴⁵ Our data set has identified more than twice as many unique glycosylation sites using just one technique, namely the hydrazide capture, but extending the analysis to the cytosol and not limiting it to the membrane fraction. The reason for this experimental design was as follows. First, it has been our experience that invariably all cell fractionation techniques remain crude methods from a proteomics perspective, enriching but never fully separating all cell compartments. Second, glycoproteins in the membrane fraction pellet may be harder to resolubilize and thus might be lost in a proteomics analysis. Finally, all proteins that are targeted to the membranes need to be synthesized in the ribosomes, transported to the endoplasmic reticulum where they are being glycosylated to be then transported to their final destination. One should therefore expect to identify these glycoproteins also in the cytosolic fractions.

3.2. Glycosylation Site Motif

To further characterize the captured glycosylated peptides, we determined the amino acid composition of the glycosylation motifs. The unique sequences of all peptides containing a deamidated N were submitted to WebLogo³⁸ and the resulting sequence atlas is shown in Figure 2. In the membrane and cytosolic fraction, 75% and 70%, respectively, showed the NXT motif and the remainder had an NXS motif. This is in agreement with previous reports.⁴⁶

3.3. Identified Glycoproteins

Overall, 106 glycoproteins were identified in this study. In the membrane fraction 57 glycoproteins were identified with 111 N-glycosylation sites, and in the cytosol fraction 95 glycoproteins were found with 245 N-glycosylation sites (Tables S1 and S2, Supporting Information). Forty-six proteins were common to both fractions. More glycoproteins were identified in the cytosol fraction compared to the membrane fraction. This could be due to the conditions used for oxidation since a high concentration of sodium periodate (>2.5 mM) can lead to increased cell death.²³ In the controls of the membrane and cytosolic fraction, zero and two glycosylation sites were

Figure 2. NXS/T motifs identified in the (A) membrane and (B) cytosolic fractions.

identified, respectively (Tables S1 and S2, Supporting Information). The very low number of glycosylation sites in the controls indicated only minor unspecific interactions between unoxidized proteins and the resin. Overall, exceptional enrichment was observed when the enriched membrane fraction (111 glycosylation sites) was compared to an unenriched membrane sample (6 glycosylation sites) (Table S1, Supporting Information). The enrichment was even higher in the cytosolic fraction, where 245 glycosylation sites were identified in the enriched sample vs five in the unenriched samples (Table S2, Supporting Information).

For comprehensiveness, the membrane and cytosolic nonglycopeptide and wash fraction were also analyzed and contained 285 proteins and 316 proteins, respectively (Tables S3 and S4, Supporting Information). When filtered against UniProt for their known glycoproteins, 34 glycoproteins were identified in the membrane fraction and 106 glycoproteins were in the cytosolic fraction (Table S3 and S4, Supporting Information). The identification of these glycoproteins was

primarily based on the presence of the nonglycosylated peptides.

3.4. Novel Glycosylation Sites

Among others, novel glycosylation sites have been identified for CD 276 antigen and mesothelin. CD 276 antigen has previously been identified in a global proteomics study focusing on the N-glycoproteome of human liver,⁴⁷ where the N189 glycosylation site was detected in the peptide GYPEAEVFWQ-DGQGVPLTGN*VTTSQM^{ox}ANEQGLFDVHSVLR of the splice isoform 2. In a recent HeLa study, N104 and N215 were found to be glycosylated (LLAQGN*ASLRL and VVLGAN*GTYSY).⁴⁵ Here, we report N104 and N215 and an additional site at N91 (GSAYAN*RTALF). Although it has long been known to be an N-linked glycoprotein, so far no glycosylation sites were identified for mesothelin.^{48–50} We identified all of the three potential glycosylation sites at N388 in DIRKWN*VTSLE, at N496 in AFQNMN*GSEYF, and N523 in ALSQQN*VSMDL. The MS/MS spectrum of the corresponding peptide ALSQQN*VSMDLATFMK is shown in Figure 3A. We also identified two glycosylation sites in the VDN*ITDQFCN*ASVDPACVR peptide of Niemann-Pick C1 protein (Figure 3B). Both glycosylation sites of the Niemann-Pick C1 protein are located in the cysteine rich luminal loop at N961 and N968.^{51,52}

3.5. Deglycosylation

When using the hydrazide capture method it is necessary to enzymatically cleave the glycans of the peptides to recover the deglycosylated glycopeptides. During deglycosylation, asparagine (N) will be converted to aspartic acid (D), and the mass difference between a non- and deamidated glycopeptide will therefore correspond to the mass difference between asparagine and aspartic acid (+0.98 Da). When the deglycosylation is performed in H₂¹⁸O, the mass difference between a non- and deamidated glycopeptide will increase to +2.98 Da. In this study the deglycosylation was carried out in both H₂¹⁶O and H₂¹⁸O. The mass differences of five representative N-linked glycopeptides deglycosylated in H₂¹⁶O and H₂¹⁸O are shown in Table 2. As seen from Table 2, the differences correspond to

Figure 3. (A) MS/MS spectrum of novel glycosylation site (#523) identified in peptide ALSQQN*VSMDLATFMK of mesothelin using ¹⁸O–H₂O resulting in a mass shift of 3 Da. (B) MS/MS spectrum of novel glycosylation sites (#961 and #968) identified in peptide VDN*ITDQFCN*ASVDPACVR of Niemann–Pick C1 protein (* indicates glycosylation site).

Table 2. Mass Difference of Five Representative N-Linked Glycopeptides Identified in HeLa Cells Deglycosylated in H₂¹⁶O and H₂¹⁸O, Respectively

peptide ^a	mass without deglycosylation	protein	deglycosylation in H ₂ ¹⁶ O		deglycosylation in H ₂ ¹⁸ O	
			mass (Da)	Δ (Da)	mass (Da)	Δ (Da)
(R)QTPEYQN*ESSR(N)	1337.5848	lysosomal acid phosphate	1338.5622	0.9774	1340.5730	2.9882
(K)DFEDLYTPVN*GSIVIVR(A)	1935.9941	transferrin receptor protein 1	1936.9711	0.9770	1938.9825	2.9884
(K)AFN*STLPTMAQMEK(A)	1567.7374	CD44 antigen	1568.7214	0.9840	1570.7257	2.9883
(R)TALFPDLLAQGN*ASLR(L)	1685.9100	CD276 antigen	1686.8941	0.9841	1688.8983	2.9883
(R)RPPLAELALN*LSGSRLEVR(A)	1663.9369	trophoblast glycoprotein	1664.9209	0.9840	1666.9252	2.9883

^a* indicates the N-linked glycosylation site.

the theoretical values and hence confirmed the peptides to be N-linked glycopeptides.

To investigate the effectiveness of the ¹⁸O-labeling procedure, we determined the percentage of deamidations that were found in the NXS/T motifs with a mass difference of 2.98 Da versus 0.98 Da in the enriched glycopeptides. More than 90% of the deamidated asparagines identified with a mass difference of 2.98 Da had the NXS/T motif when the samples were deglycosylated in H₂¹⁸O as shown in Figure 4 for proteins from the membrane and cytosolic fractions. Fewer than 10% were identified with a mass difference of 0.98 Da and a

glycosylation motif, indicating a very high incorporation of H₂¹⁸O.

The reason for identification of glycosylation sites with only a 0.98 Da mass difference may be due to not fully labeling, addition of PNGase F or N-glycanase that was not dried or back-exchange when dissolving the sample in 0.2% formic acid prior to nanoLC-MS/MS analysis. Finally, although unlikely, a small portion may also be due to spontaneous deamidation at proper glycosylation motifs.

An additional confirmation of N-linked glycopeptides can also be obtained through MS/MS analysis. As an example, the MS/MS spectra of (R)TALFPDLLAQGN*ASLR(L) identified in isoform 1 of CD 276 antigen deglycosylated in H₂¹⁶O and H₂¹⁸O are shown in Supplementary data, Figure S1. All of the b- and y-ions corresponding to asparagine have a mass difference of 2 Da between the H₂¹⁶O-deglycosylated and H₂¹⁸O-deglycosylated form of the peptide confirming successful deglycosylation and identification of N-linked glycosylation sites.

3.6. Enzymatic versus Chemical Deamidation

As previously reported, asparagine can spontaneously deaminate to aspartic acid, even without the presence of deglycosylating enzymes.^{53,54} Therefore, additional measures are necessary to avoid false positive glycosylation site identification. ¹⁸O-labeling has previously been used successfully to further define the glycosylation sites.⁵⁵ One might still argue that chemical deamidation might have happened in the presence of H₂¹⁸O and thus is indistinguishable from an enzymatic deamidation.

To investigate this possibility, we determined the false discovery rate (FDR) of the glycosylation sites by comparing the number of deamidated N sites with NXS/T motif vs random deamidation (without motif) between the enriched glycopeptide fractions of the oxidized (enriched) and unoxidized (control) samples collected. Since the unoxidized sample should not bind to the hydrazide resin, no enzymatic deglycosylation should occur in this sample.

As shown in Figure 5, we found zero deamidations in the membrane control samples and in the cytosol controls, two had an NXS/T motif (Tables S1 and S2, Supporting Information). This compares to a total of 199 and 349 redundant (resulting in 111 and 245 unique) NXS/T sites identified in the membrane and cytosol samples. From these data, we can estimate that our FDR for glycosylation site determination is less than 1%, indicating a very low number of false glycosylation site identifications.

Figure 4. Percentage of deamidated asparagine (N) identified with a mass difference of 2.98 and 0.98 Da with (dark gray) and without (light gray) the NXS/T motif in (A) membrane and (B) cytosolic fractions after deglycosylation in H₂¹⁸O. Replicates (Rep) are shown separately.

Figure 5. Number of deamidated asparagine (N) identified with (striped pattern) and without (gray) the NXS/T motif in (A) membrane and (B) cytosolic fractions of the enriched glycopeptide samples and controls. Each replicate is shown separately. The FDR of false glycosylation site determinations was determined to be less than 1%.

3.7. Enrichment of the N-Linked Glycosylation Sites in the Enriched Glycopeptide Fraction

To determine the enrichment of the hydrazide capture for glycosylated proteins, the percentage of the deamidated N with NXS/T glycosylation motif in the enriched glycopeptide fraction was compared to the unenriched sample as well as to known glycoproteins listed in UniProt. As shown in Figure 6, significant enrichment for glycosylation sites (Figure 6A) and glycoproteins (Figure 6B) was observed using the hydrazide capture method.

3.8. Transmembrane Domain (TMD) Analysis

To test whether the identified proteins from the membrane and cytosol fractions were indeed mostly transmembrane domain containing proteins, we separately analyzed the enriched proteins with identified glycosylation sites with TMHMM. As shown in Figure S2 (Supporting Information), the percentage of identified TM domains in membrane and cytosolic fraction is similar with ca. 50% containing 1 TMD, and the remainder containing up to 14 TMDs. In both cases, ca. 30% of the identified proteins were not predicted to contain a TMD. In addition, five proteins (bone marrow stromal antigen 2, cadherin-13, folate receptor 1, CD59 glycoprotein and

Figure 6. (A) Number of NXS/T motif containing glycosylation sites and (B) number of glycoproteins in enriched vs unenriched membrane (striped pattern) and cytosolic fractions (gray).

mesothelin) are predicted to have glycosylphosphatidylinositol (GPI) anchors.⁵⁶

4. FUNCTIONAL ANALYSIS

Glycoproteins identified in the enriched glycopeptides fraction were subjected to further bioinformatic analysis. While our experimental design enriched glycoproteins by at least 100-fold, it was not meant to elucidate entire signaling cascades. Rather, it was designed to capture N-linked glycoproteins, many of which are known to be involved in cancer-related signaling. DAVID analysis indicated a significant enrichment for the following cancer-related KEGG pathways: focal adhesion (p value 2.91×10^{-04}), cell adhesion molecules (p value 5.49×10^{-05}), ECM–receptor interaction (p value 1.17×10^{-07}), regulation of actin cytoskeleton (p value 0.023801012), lysosome (p value 1.95×10^{-20}), small cell lung cancer (p value 8.20×10^{-04}), pathways in cancer (0.029302846), and sphingolipid metabolism (p value 0.014827196). (Tables S7 and S8, Supporting Information).

4.1. Cell Adhesion Molecules (CAMs) and Extracellular Matrix (ECM)–Receptor Interaction

For a cancer cell to metastasize, it first needs to adhere to the extracellular matrix and surrounding stromal cells via cell adhesion molecules. Therefore, CAMs have been associated with tumor progression and metastasis and have been proposed as biomarkers.^{57–63} In general, they can be categorized into integrins, cadherins, and members of the immunoglobulin superfamily.

Integrins are transmembrane glycoproteins that are involved in cell-matrix and cell-cell interactions. Integrins thus play a critical role in adhesion, cytoskeletal remodeling, and cell motility. Interestingly, integrin α -6 (CD49f) has been identified as the primary receptor of human papillomavirus (HPV), known to have caused cancer in HeLa.^{2,64} In addition to integrin α -6 mentioned above, cancer stem cell marker CD44, a heparan sulfate proteoglycan, may also be a receptor for human papillomavirus.⁶⁵ When alternative splicing and glycosylation sites are taken into account, at least 20 variants of CD44 with different functions have been described so far. In fact, CD44 is the most often described biomarker.⁶⁶ CD44 signaling is a highly orchestrated process.⁶⁷ Shedding of the ectodomain of CD44 seems regulated through proteolysis by activated protease ADAM17, which was also detected in this study, and is activated by protein kinase C (PKC) and the small GTPase Rac. This process enables migration of the cell. At the same time, the intracellular domain is released into the nucleus upon shedding and, there, regulates further gene transcription.⁶⁸ CD44 isoforms associate with glycoproteins tetraspanins and N-cadherin (EpCAM), both identified in this study. CD44 and N-cadherin are regulated by the β -catenin-dependent Wnt signaling pathway, a prominent target for cancer therapies.^{69,70}

Cadherins are calcium-dependent adhesion molecules that mediate cell motility of cancer cells. Specifically, N-cadherins have been implicated in metastasis by enabling cancer cells to migrate through the endothelial layers into the bloodstream to reach their target organs.⁷¹ In contrast to cadherins, the immunoglobulin superfamily CAMs are cation-independent. Similar to cadherins, they play a key role in tumor cell invasion enabling dynamic cell-cell communication.⁷² Specifically, activated leukocyte-cell adhesion molecule ALCAM (CD 166) has been identified as cancer stem cell marker.^{63,73}

CD 276 antigen is a ubiquitous surface protein that has previously been defined as a neuroblastoma biomarker.⁷⁴ Interestingly, it was found that CD276 antigen may repress NK cell mediated lysis of the tumor cells.⁷⁴ Thus, CD276 antigen may be an interesting therapeutic target where an effective reduction of the antigen's protective role of the tumor cells could increase antitumor efficacy of anticancer drugs.

Although not identified by the KEGG analysis, several other proteins are associated with cellular adhesion: mesothelin,⁷⁵ which has previously been identified as a biomarker in cancer; the cell adhesion molecule neuropilin (aka stromal cell derived receptor 1);⁷⁶ podocalyxin, known to be implicated in adhesion, cell morphology and cancer progression;⁷⁷ CD97 antigen,^{78,79} which has been associated with increased motility and invasiveness, that is the ability to break through endothelial cell barriers and attach to distant organs; galectin-3-binding protein, involved in integrin mediated cell adhesion and fibronectin matrix remodeling in tumor cells and basigin^{80,81} (an extracellular matrix metalloproteinase inducer EMMPRIN), which is known to be enriched on tumor cell surfaces. It stimulates collagenase and thus contributes to tumor progression.^{82,83}

4.2. Focal Adhesion and Regulation of Actin Cytoskeleton

Cell proliferation, survival, migration, and invasion are also regulated by focal adhesions, which are specialized complexes that anchor actin filaments to integrins. Integrins either bind to the RGD motif (laminin, fibronectin, and vitronectin) or the DGEA and GFOGER motifs (collagen). Downstream signaling

including focal adhesion kinase enables reorganization of the actin cytoskeleton and thus allows cells to change their shape and migrate, a key process during metastasis.

In addition to the integrins and laminins, epidermal growth factor receptor (EGFR) was also identified in this study. EGFR has been implicated in focal adhesion, likely mediated through two members of the tensin family.⁸⁴ Mutations leading to EGFR overexpression are involved in many cancers and are targets for a number of anticancer therapies.⁸⁵ In cervical cancer, EGFR has also been suggested as a biomarker.⁸⁶

4.3. Lysosome

Changes in lysosomal trafficking and increased expression of cathepsins are associated with tumor invasion and metastasis. Particularly, secreted cathepsins are involved in ECM degradation, which in turn enables tumor invasion. Lysosome-associated membrane glycoproteins LAMP1, 2, and 3 have been implicated in tumor progression and metastasis.^{87–89}

The minor lysosomal membrane protein Niemann–Pick C1 was initially discovered to be involved in intracellular trafficking of cholesterol. More recent work also proposed a vesicle-mediated pathway for lysosomal drug clearance. In contrast to drug-sensitive cell lines, multidrug resistant (MDR) cell lines were associated with loss of NPC1 activity and showed lysosomal drug accumulation.⁹⁰ A mutation in the Niemann–Pick C1 protein may be the reason for the Niemann–Pick disease type C1 to develop but can also be acquired in MDR cancer cells. Interestingly, asparagine to serine mutations have been observed for both N961 and N968.^{51,52,91} Since serines cannot accept N-glycosylations, this will likely alter the glycosylation pattern and thus the function of this protein. Monitoring glycosylation sites using the hydrazide capture method in combination with LC–MS/MS could therefore be a useful diagnostic tool for MDR development and, in a different context, Niemann–Pick disease type C1.

The multifunctional cation independent mannose-6-phosphate receptor (insulin growth factor 2 receptor, IGF2R, CD222) interacts with putative phospholipase B-like glycoprotein, both identified in this study,⁹² and binds to heparanase, which in turn cleaves heparan sulfate, thus essentially enabling ECM degradation.⁹³ Together with Cathepsin D, IGF2R has been suggested as a biomarker⁹⁴ and correlates with tumor progression.⁹⁵

4.4. Pathways in Cancer

In addition to the cancer-related proteins described above, KEGG analysis identified two additional glycoproteins as cancer-related: The facilitated glucose transporter (GLUT1)/solute carrier family 2, member 1 protein (SLC2A1), and HSP90B1; heat shock protein 90 kDa β . SLC2A1 is upregulated in radioresistant and chemoresistant hypoxic cells and has thus been suggested as a prognostic biomarker for cancer therapy.⁹⁶

Owing to the stress that cells experience when they become cancerogenic, chaperones like the HSP90B1 have been identified as important drug targets.⁹⁷ In addition, HSP90B1 was recently proposed as a prognostic biomarker for distant metastasis.⁹⁸

In addition to those proteins described above, some additional cancer biomarker are of note, e.g., CD 109 antigen, which is upregulated in squamous cell carcinomas, but not in adenocarcinoma and thus may serve as a diagnostic marker to differentiate these cancers;⁹⁹ CD97 antigen, which was proposed as one of several biomarkers that are upregulated in

invasive cervical carcinomas but not in normal cervical keratinocytes;¹⁰⁰ antiviral defense protein CD317 (bone marrow stromal antigen 2, tetherin), which inhibits viral release from cell surface;¹⁰¹ major and minor histocompatibility class proteins, which are proposed as diagnostic markers for cervical cancer;¹⁰² cancer biomarker trophoblast glycoprotein;¹⁰³ seizure 6-like protein, which has an increased expression in various lung cancers, but has not yet been investigated in cervical cancers,¹⁰⁴ and inhibitor of NF κ B kinase-interacting protein, which is a target of tumor suppressor p53.¹⁰⁵

5. CONCLUSIONS

In this study, the hydrazide capture method was used to enrich and profile N-linked glycosylation sites in cytosolic and membrane-associated protein HeLa cells. We have demonstrated that the hydrazide method selectively enriches at least 100-fold N-linked glycoproteins in contrast to unenriched samples. Additionally, the degree of unspecific interactions was determined to be very limited by comparison with nonoxidized samples which served as control. Hence, the enrichment of glycoproteins is due to an interaction with hydrazide and not due to any secondary interactions. By extending this methodology to the cytosolic fractions, we identified 268 unique glycosylation sites with an NXT/S motif, more than twice as many as previous studies. Both known and novel glycosylation sites were determined in a total of 106 glycoproteins. Many of these glycoproteins are cell surface proteins responsible for cell–cell or cell–matrix recognition, binding and migration. In addition, signaling cascades are activated by a number of enzymatic, mostly proteolytic processes. Cell adhesion, migration and proteolysis are highly associated with tumor invasiveness. In numerous studies, glycoproteins involved in these processes have been suggested to be either prognostic or diagnostic cancer biomarkers. We envision that this method could be applied in cancer tissue differential diagnostics and further developed to achieve best therapeutic outcomes in personalized medicine.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and supplementary figures, data tables, and spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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