Aberrant sialylation and fucosylation of intracellular proteins in cervical tissue are critical markers of cervical carcinogenesis

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Abstract. Numerous studies have suggested that increased sialylation and fucosylation levels are signs of cancer progression. The majority of studies have focused on cell surface and bloodstream glycosylation changes associated with cancer progression, while little attention has been paid to changes in the glycosylation of cytosolic proteins. We compared the mannosylation, sialylation and fucosylation levels of cytosolic proteins obtained from human cervical tissues without neoplastic lesions vs. with cancer, using lectin blot and enzyme-linked lectin assay (ELLA) systems. There were no quantitative differences in mannosylation levels between the cytosolic proteins of normal and cancer tissues. However, we found markedly reduced sialylation (P<0.001) and fucosylation (P<0.01) in the proteins of cancer tissues. The ELLA system for detecting sialylation had extremely high sensitivity (91-100%) and specificity (82-100%) in distinguishing normal and cancer tissues. Thus, the changes in the glycosylation of cytosolic proteins during carcinogenesis of the cervix are quite different from previous observations concerning glycoconjugates in the bloodstream or on the cell surface. We suggest that changes in the glycosylation of intracellular proteins may be useful markers of the development of cervical cancer.

Introduction

Cervical cancer is a major cause of female mortality worldwide, and almost all cases are caused by human papillomavirus (HPV) infection (1). Cervical cancer develops in ~500,000 women annually and is responsible for 250,000 deaths (2). The prevalence of HPV infection among women is known to be highest in those aged 20-24 years (44.8% of prevalence) (3). Most HPV infection resolves spontaneously. However, cervical cancer develops over a period of 12-15 years when the HPV infection persists (4). Two oncogenic proteins, E6 and E7, of HPV cause genetic instability and uncontrolled growth of epithelial cells and can lead to their immortalization (5,6). The Papanicolau (Pap) smear test has been used for more than 50 years to detect asymptomatic women with cytological abnormalities (7), and women with abnormal cytology are referred for subsequent tests such as colposcopy and directed biopsy (gold standards).

Screening and prevention are the most critical factors in reducing the incidence of cervical cancer. Current cytological screening programs using the Pap test have significantly reduced the incidence of cervical cancer (7). Despite the success of the Pap test, its low sensitivity remains a problem, and several auxiliary techniques have been proposed to increase the fidelity of diagnoses (8). Ki-67 and p16 have been identified as molecular biomarkers of cytological status, as their overexpression is associated with oncogenic potential and cancer progression (9,10). In addition, mini chromosome maintenance protein (MCM), cell division cycle protein 6 (CDC 6) and squamous cell carcinoma antigen (SCC) have been proposed as useful molecular markers (11-13).

Genomic and proteomic approaches are now the preferred routes to new diagnostic tools in the cervical cancer research field (14,15). Biomarkers distinct from proteins and gene sequences would provide additional approaches to the diagnosis of cervical cancer. Accumulating evidence indicates that glycosylation plays a pivotal role in disease progression and that aberrant glycosylation is a reliable marker of carcinogenesis (16,17). Increased sialylation and/or fucosylation of cancer tissues are thought to be associated with tumorigenicity, invasiveness and metastatic ability (18,19).

Little attention has been paid to changes in the glycosylation of intracellular proteins in relation to cancer development. In the present study, we examined such changes for the first time. Using lectins, we compared the sialylation, fucosylation and mannosylation levels of cytosolic proteins in normal and cancer tissues from the human cervix. We showed that the glycosylation changes in intracellular proteins can provide a unique means of identifying carcinogenesis.

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Key words: biomarker, cervical cancer, papillomavirus, glycosylation, sialic acid

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Materials and methods

Clinical samples. Eleven cervical cancer tissue samples were obtained from patients who underwent surgical resection for squamous cell carcinoma or adenocarcinoma at the Ewha Womans University Mokdong Hospital (Seoul, Korea). Ten cervical normal tissue samples without neoplastic lesions were obtained from the same source. Pathology, age, chemotherapy and radiotherapy history, infecting HPV type and size of the cancer of the women with normal cytology and cervical cancer are presented when relevant in Table I. All specimens were immediately frozen and stored at -80°C for subsequent analysis. The procedure for resection of cervical biopsies was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital.

Preparation of cytoplasmic tissue lysates from the biopsies and paraffin blocks of the cervix. The cervical biopsies were weighed and mixed with lysis buffer [100 mM Tris-Cl (Duchefa Biochemie B.V, Haarlem, The Netherlands) pH 7.4, 150 mM NaCl (Duchefa), 1 mM EDTA-Na (Duchefa) and 1% Triton X-100 (USB Corp., Cleveland, OH, USA)]. The mixtures were disrupted with a Dounce homogenizer (Duran Group GmbH, Mainz, Germany). To obtain the soluble protein fraction, each lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was collected. The protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). To obtain lysates from paraffin blocks, deparaffinization was carried out as previously described (20). The deparaffinized cervical tissue was detached and prepared as described above.

SDS-PAGE and lectin blotting of cervical tissue lysates. SDS-PAGE was performed as previously described (21), and the fractionated proteins were visualized with silver staining. Tissue supernatants were mixed with Laemli’s loading buffer (21), fractionated on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (ATTO Corp., Tokyo, Japan). The membrane was blocked overnight with 2.5% oxidized bovine serum albumin (oBSA) in TBS-T. oBSA was prepared as follows. BSA (Bioworld, Dublin, OH, USA) was dissolved in 10 mM sodium metaperiodate (Sigma, St. Louis, MO, USA), maintained at 4°C for 1 h and dialyzed against Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 16 h at 4°C. The PVDF membrane was reacted with biotinylated concanavalin A (ConA), Sambucus nigra lectin (SNA) or Aleuria aurantia lectin (AAL). All lectins were purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Proteins bound to the lectins were detected with HRP-conjugated streptavidin (Pierce) and developed on X-ray film (Kodak, Rochester, NY, USA) using a chemiluminescence reagent kit (AbClon, Seoul, Korea). Lectins and streptavidin were prepared in 0.25% oBSA in TBS-T.

Enzyme-linked lectin assays (ELLA) of the cervical tissue lysates. ELLA was performed as previously described with modifications (22). A 96-well microplate (Greiner Bio-One, Frickenhausen, Germany) was coated overnight with 5, 2.5 or 1.25 μg of tissue lysate protein per well at 4°C and blocked with 2.5% oBSA in TBS-T for 4 h at room temperature (RT). Thereafter, the wells were incubated with biotinylated ConA, SNA or AAL for 1 h at 37°C followed by HRP-conjugated streptavidin for 40 min at 37°C. The reactions were developed with o-phenylenediamine (Sigma) and measured at 492 nm.

Statistical analysis. The statistical significance of differences between groups was determined by two-tailed Student’s t-tests. P-values <0.05 were considered to indicate statistically significant results. Sensitivity and specificity were calculated as follows: Sensitivity = number of true positives x 100/number of true positives + number of false negatives; Specificity = number of true negatives x 100/number of true negatives + number of false positives. The cut-off value for each assay was set as follows: (Median value of normal group + median value of cancer group)/2.

Results

Banding patterns of intracellular cervical proteins on lectin blots. It is well-known that ConA, SNA and AAL react with mannoses, α2,6-linked sialic acids and fucoses, respectively (23,24). Fig. 1 shows representative patterns of the cytoplasmic proteins after SDS-PAGE and visualization by silver staining and lectin blotting. To obtain representative banding patterns, 10 individual lysates of normal cervix tissues were combined, as were lysates of 11 cervical cancer tissues (Table I). Fig. 1A shows that the protein composition of the cervical cancer tissues was more varied than that of the normal cervix tissues. The normal and cancer tissues contained glycoproteins of various molecular masses that reacted with ConA (Fig. 1B). It is thought that highly abundant mannosylated proteins are detected because mannose is the main component of mammalian N-glycans (25). There was no quantitative difference between the normal and cancer tissues regarding the reactivity of their proteins with ConA (Fig. 1B). In contrast, the lysates of the cancer tissues had markedly reduced reactivity to SNA (Fig. 1C) and the banding pattern after reaction with AAL was distinct from that of the normal tissues (Fig. 1D). Concerning AAL binding, a 48-70 kDa diffuse band was a major band in the cervical cancer tissue preparation (open arrow) while a tight 50-kDa band was the major band formed by normal tissues (filled arrow). These results indicate that there were significant changes in the α2,6-sialylation and fucosylation of cytosolic glycoproteins in the cancer tissues.

To compare the sialylation and fucosylation levels of the intracellular proteins in more detail, we analyzed the reactivities of proteins from individual normal and cancer tissues to SNA and AAL. Most of the cervical cancer specimens showed significantly reduced SNA binding (Fig. 2A), and there was little variation between the individual normal or cancer specimens. The proteins of the individual cancer tissues also tended to bind less AAL (Fig. 2B). However, the individual samples of normal and cancer tissues varied considerably with respect to the banding pattern and band intensities (Fig. 2B).

Reactivities of intracellular proteins of normal and cancer tissues towards lectins in ELLA. ELLAs were carried out to compare the levels of mannosylation, sialylation and fucosylation of the cytosolic proteins in the proteins immobilized in
Table I. Information regarding the cervical biopsies from normal and cancer tissues.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Specimen no.</th>
<th>Age (years)</th>
<th>Pathology</th>
<th>Size of cancer (cm)</th>
<th>Radiotherapy prior to resection</th>
<th>Chemotherapy prior to resection</th>
<th>HPV type infected</th>
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<tr>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 treatments of RT 11 years ago</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>54</td>
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<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>49</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
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<td>6</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<td>10</td>
<td>47</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>SCC</td>
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<td>5</td>
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<td>SCC</td>
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<tr>
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<td>10</td>
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<td>SCC</td>
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<td>-</td>
<td>-</td>
<td>HPV 16</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>48</td>
<td>SCC</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>HPV 16</td>
</tr>
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</table>

RT, radiotherapy. HPV, human papillomavirus; SCC, squamous cell carcinoma.

Figure 1. Representative banding patterns of cytosolic proteins from normal and cancer tissues following SDS-PAGE and lectin blotting. Ten individual lysates from normal tissues and 11 from cancer tissues were separately combined to obtain these representative banding patterns. (A) Silver staining. Blots for (B) ConA, (C) SNA and (D) AAL, respectively. N and C refer to proteins from normal and cancer tissue, respectively. The gel labeled M shows protein markers. One microgram/well of cytosolic protein was loaded for the silver-stained gel, and 50, 250 or 500 ng of cytosolic protein per well for the lectin blots. Arrows indicate the protein amount loaded. The filled and open arrows indicate the normal tissue-specific band and the cancer tissue-specific band, respectively.
Figure 2. Banding patterns of individual specimens in the lectin blots. Results of lectin blots for (A) SNA and (B) AAL. (A) One hundred and (B) 300 ng of cytosolic proteins were loaded to investigate the reactivity of the proteins to SNA and AAL, respectively. Details of each specimen are documented in Table I. Normal, n=9; cancer, n=9. Two lectin blots (upper and bottom panel) were performed to analyze the banding patterns of 9 individual normal tissues and 9 individual cancer tissues.

Figure 3. Reactivities of cytosolic proteins to lectins in ELLA. Ninety-six-well microplates were coated with 2.5 µg of cytosolic protein/well and tested for binding of (A) ConA, (B) SNA and (C) AAL. The central line represents the median, and the top (Q3) and bottom (Q1) lines, the 75th and 25th percentiles, respectively. The top and bottom whiskers represent outliers. Normal, n=10; cancer, n=11 (panels A-C). (D) The ELLA results obtained by testing SNA and AAL binding of cytosolic proteins from the paraffin blocks. One microgram/per well of protein was used for coating. Normal, n=3; cancer, n=3 (panel D).
Recently, it has been proposed that the cellular location (30). However, their functions still remain unclear.

There are four types of cellular sialidases, and their properties and functions are thought to depend on their particular subcellular location (30). However, their functions still remain unclear. Recently, it has been proposed that the α2,3- and α2,6-terminal sialylation levels of cell surface glycoconjugates in cervical biopsies increase with the grade of squamous intraepithelial lesions (SILs) (31). In the present study, however, the reduction in sialylation levels observed in the cytosolic proteins of cervical cancer tissues was opposite to the increase noted on the cell surface (31). Therefore, it appears that the sialylation pathway of cytotoxic glycoconjugate is significantly different from that for cell surface glycoconjugates.

There is evidence that the intracellular glycosylation changes occurring during carcinogenesis are independent of those that occur on the cell surface or in the blood. Krzeslak et al (32) observed that the sialylation levels of intracellular proteins in thyroid lesions, particularly follicular and papillary carcinomas, were significantly lower than those in non-neoplastic lesions. However, there have been no subsequent confirmatory studies. In our cases, we also observed reduced sialylation and fucosylation levels in the inner part of the cervical cancer tissues. Therefore, it is evident that there are significant changes in the glycosylation of intracellular proteins in carcinomas.

Our ELLA results showed that the difference in fucosylation level in the cytosolic proteins between the normal and cancer tissues (Fig. 3C) was less than that of the sialylation level (Fig. 3B). As shown in Fig. 2B, both normal and cancer specimens showed individual variations in fucosylation levels in the lectin blots. Therefore, there is an inherent limit to the sensitivity and specificity of ELLA using AAL with whole tissue supernatants (Fig. 3C and Table I). However, this limitation may possibly be overcome by examining the fucosylation level of specific proteins. As shown in Fig. 2B, a protein band of 150 kDa was noted only in the normal tissues (filled arrow) while a 120-kDa protein band was noted only in the cancer tissues (grey arrow). Therefore, increased specificity and sensitivity may be achieved if the fucosylation of this specific protein is measured. This type of assay could also be performed using antibody-based enzyme-linked lectin assays (22).

False-negatives in conventional Pap tests and in the liquid-based Pap test have been a major hindrance to accurate diagnosis for more than five decades. Approximately 30% of new cases of cervical cancer are initially interpreted as negative (33,34). Moreover, many researchers have suggested that the sensitivity of conventional Pap testing is only ~50%, which means that the Pap test is not suitable for use as the sole approach to cervical cancer screening (7). Therefore, the development of new strategies to complement the cytological test is a high priority. The ELLA using SNA developed in the present study showed 90-100% sensitivity and 82-100% specificity (Table I). Approximately 10-30 µg of cytosolic protein can be obtained from a Pap smear (data not shown), and our ELLA system for detecting sialylation requires 1.2-2.5 µg of protein per assay. This indicates that there is considerable potential for analyzing sialylation levels using proteins obtained from Pap tests. We anticipate that future investigation of the combination of the Pap test and our ELLA system could substantially reduce the proportion of false-negative results.

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References


