Down-regulation of insulin-like growth factor binding protein-5 (IGFBP-5): Novel marker for cervical carcinogenesis

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To better understand the underlying pathways of cervical carcinogenesis, cDNA microarray analysis was performed on 2 sets of squamous cell carcinomas (SCCs) and their adjacent normal squamous epitheliums. Consistently altered expression was detected for 32 genes. Real-time RT-PCR analysis was conducted on a selected subset of these genes (S100A2, GPCA, p72, IGFBP-5, TRIM2 and NAB2) for 14 additional SCCs and 10 normal epithelia. This found that, of the 6 candidate genes, only the insulin-like growth factor binding protein-5 (IGFBP-5) mRNA was generally and significantly under-expressed in SCCs (p < 0.001). All normal cervical epithelia (30 of 30) stained positively for IGFBP-5 protein, with 70% showing strong staining, whereas 65% (17/26) of SCC had complete loss of IGFBP-5, and only 8% (2/26) SCC retained strong expression (p < 0.001). Immunohistochemistry of premalignant cervical intraepithelial neoplasia (CIN) lesions shows a significantly weaker or negative staining in advanced CIN3 lesions compared with normal squamous epithelia (p = 0.001). This is the first study to show that down-regulation of IGFBP-5 protein correlates with cervical carcinogenesis and does so at a preneoplastic stage.

Key words: squamous cell carcinoma (SCC); cervical intraepithelial neoplasia (CIN); human papillomavirus (HPV); insulin-like growth factor binding protein-5 (IGFBP-5); cDNA microarray; real-time RT-PCR; immunohistochemistry

Uterine cervical cancer is the second most common malignancy among women worldwide,1 with an estimated 470,000 new cases per year and mortality rate which ranks third worldwide, with the highest rates occurring in developing countries.2 Squamous cell carcinomas account for the majority of cervical cancers, followed by adenocarcinomas which comprise 10–18% of cases, and the remainder of tumors consists of a few rare types.3,4 The squamous cell carcinomas and adenocarcinomas are mainly caused by infection with one of the high-risk types of human papillomaviruses (HPV), exemplified by the HPV-16 and -18 strains; so called high-risk because of their consistent detection in high-grade squamous intraepithelial cervical lesions and carcinomas.4,5 The E6 and E7 viral oncoproteins of high-risk HPV play critical roles in initiating cervical carcinogenesis by binding to and degrading or inactivating the cellular tumor suppressor proteins p53 and Rb.6–8 These viral-oncogenic properties have provided the general basis for the extensive investigations of the role of HPV in carcinogenesis of the HPV-infected cervix, demonstrating how they subvert key cell cycle and regulatory processes to transform and immortalize the host cell.1,3,5

It follows that alterations of protein and mRNA expressions in cervical cancer have been investigated specific to the HPV infection; however, the more global profile of gene expression variation between cervical cancer and normal squamous epithelium is still largely unknown. The exploration of cancer-specific up- and down-regulation of genes based on expression profiling will be greatly beneficial in finding new cervix-specific biomarkers to detect, monitor and predict disease progression, and to discover better therapeutic targets for this prevalent and deadly cancer.

The microhybridization-based gene-expression cDNA microarray assay makes possible the parallel hybridization to tens of thousands of different gene probes simultaneously, thus providing for a relatively unbiased mRNA expression profiling of tissues.16–18 We have used this technique to investigate the gene expression profiles of squamous cell cervical cancers, and we have preliminarily identified altered cervical-cancer-specific expression patterns for 32 genes. We sought to validate the significance of the differences in mRNA expressions of 6 likely candidate genes in a larger pool of SCC by the real-time reverse transcription polymerase chain reaction (RT-PCR) assay. Finally, we have convincingly shown by immunohistochemistry that the significant loss of IGFBP-5 mRNA expression extends to the protein level and occurs early in the process of cervical carcinogenesis.

Material and methods

Materials

The tissues used in the present study were obtained from patients at the Osaka University Hospital after obtaining informed consent. For the cDNA microarray analysis we obtained frozen samples from 2 cases of uterine cervical squamous cell carcinoma, both of the nonkeratinizing type and classified as FIGO Stage Ib. Carcinoma lesions and adjacent normal cervical squamous epithelium of the 2 cases were identified, manually dissected and snap-frozen in liquid nitrogen within 30 min of excision. For the real-time RT-PCR analysis, frozen specimens were obtained from 14 nonkeratinizing squamous cell carcinomas of the cervix, which ranged from FIGO Stage Ia to IIIa. Ten frozen specimens of normal squamous epithelium were also obtained from hysterectomy cases conducted due to leiomyoma, endometriosis or other carcinomas, including endometrial carcinomas and ovarian carcinomas, for which cervical tissue was not involved. Each lesion was dissected and snap-frozen in liquid nitrogen as described above.

Formalin-fixed paraffin-embedded tissue specimens of 26 cases of cervical squamous cell carcinomas, which ranged from FIGO Stage Ia to IIIb, were collected for immunohistochemical investigation. Seven of these 26 cases were of the keratinizing-type and the remaining 19 cases were of the nonkeratinizing type. For comparative immunohistochemical analysis, 30 specimens of normal cervical squamous epithelium were obtained from hysterectomy cases due to benign or malignant diseases in which the cervical tissue was not involved. For the analysis of cervical lesions, 23 cases of squamous metaplasia, 20 cases of cervical intraepithelial neo-
plasia I (CIN1), 27 cases of CIN2 and 21 cases of CIN3 were obtained from biopsy or hysterectomy cases.

**HPV sub-typing**

The subtype of HPV infection of the 2 carcinomas used for the microarray analysis was determined by a previously described method of PCR-RFLP analysis.19

cDNA preparation

Total RNA from each specimen was extracted using Trizol (Invitrogen Corp., Carlsbad, CA), chloroform and isopropyl alcohol. The template cDNA was synthesized from total RNA using a cDNA Cycle Kit (Invitrogen) with random primers. The reverse transcription reaction was done at 42°C for 60 min followed by heating at 95°C for 3 min.

cDNA microarray analysis

Affymetrix Gene Chip U95 arrays (Affymetrix, Santa Clara, CA), containing approximately 12,000 known genes and 48,000 expressed sequence tags (ESTs), were used to compare the mRNA expression profile of each squamous cell carcinoma with that of its adjacent normal cervical squamous epithelium using the GeneChip Software System (Gene Logic, Gaithersburg, MD). Fold-change analysis, in which the ratios of the average of the gene expression intensities of the relevant gene were computed and in which the ratios were reported as the “fold-change” (up or down), was performed. A 2 or greater change of intensity for the malignant lesion compared with the normal squamous epithelium of the same case was considered to be an up-regulated or down-regulated expression of the gene. Genes for mRNAs commonly up- or down-regulated in both squamous cell carcinomas were selected for further study.

Gene selection

To determine whether the differences in gene expression profiles detected in the cDNA microarray analysis of only 2 cases were more widely significant, a working subset of 6 known genes was selected from the larger group of 32 candidate markers based upon their Gene Ontology (GO) classification.20 The 6 selected genes were involved in cell proliferation, cell cycle progression, DNA replication or repair, cell differentiation, apoptosis or metastasis.

**Real-time RT-PCR analysis of marker candidates**

The real-time RT-PCR analysis was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes, which contained 6-FAM (6-carboxyfluorescein) (Applied Biosystems) as the reporter fluorophore, were designed for each specific cDNA using the TaqMan Assay-on-Demand gene expression system (Applied Biosystems). The primers and the probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, containing VIC (proprietary, Applied Biosystems) as the reporter fluorophore, were used as the internal control for gene expression. The 50 μl of PCR mixture consisted of 1 μg of template cDNA, 2.5 μl of 20X target primers and probe (final concentration; 900 nM of each primer and 200 nM of probe), 2.5 μl of 20X control primers and probe (final concentration; 900 nM of each primer and 200 nM of probe) and 25 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems). The amplification conditions were: 2 min at 50°C, 10 min at 95°C, followed by a 2-step cycle of 95°C for 15 sec and 60°C for 60 sec, for a total of 50 cycles. The quantitative values were obtained from the threshold cycle (Ct) number at which the increase in fluorescent signal associated with an exponential increase of PCR products was detected. The standard curves for cDNA of each target gene and GAPDH were generated using serially diluted solutions and the amount of target gene expression was calculated from these standard curves. The corresponding mRNA expression levels of each gene were shown as ratios to those of the GAPDH gene. The Mann–Whitney U test was used for the assessment of the relationship of mRNA expressions between the carcinoma and its associated normal epithelial tissue. The p-values of <0.05 were considered to be statistically significant.

**Immunohistochemical staining**

Formalin-fixed, paraffin-embedded tissue sections were cut at 4 μm and placed on poly-L-lysine-coated glass slides (Matsunami, Osaka, Japan). The sections were deparaffinized in xylene and dehydrated in graded alcohols. Antigen retrieval was done in a citrate buffer (10 mM, pH 6.0) at 95°C for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Immunohistochemical staining was performed using an indirect avidin–biotin immunoperoxidase method (Vector Laboratories, Burlingame, CA). After counter-staining with hematoxylin, the sections were dehydrated in graded alcohols and xylene, and then coverslipped.

The specificity of the IGFBP-5 antibody was confirmed by a blocking study. Five micrograms of recombinant human IGFBP-5 (Austral Biologicals, San Ramon, CA) was mixed with 2.5 μg of anti-IGFBP-5 antibody and incubated for 1 hr at room temperature and was centrifuged at 21,000g for 10 min. The supernatant was substituted for unblocked IGFBP-5 antibody solution and a complete immunohistochemical staining procedure was conducted as described earlier.

The staining of IGFBP-5 was microscopically evaluated and scored as follows: (+ +), over 50% of the cells demonstrated strong staining; (+), strong staining was observed in less than 50% of the cells, or, even if over 50% of cells were stained, the intensity of the staining was weak; (−), no stained cells were identified in the tissue.

The χ² test was used for the assessment of the relationship between the staining of carcinoma and normal cervical tissue. The p-value of <0.05 was considered to be statistically significant.

**Results**

**HPV sub-typing**

The 2 nonkeratinizing-type Stage Ib uterine cervical squamous cell carcinoma samples analyzed for HPV infection status were found to have high-risk HPV-16 DNA in the 1 case and high-risk HPV-58 in the other.

cDNA microarray analysis

The vast majority of the 12,000 expressed genes and ESTs were expressed equally in both the cancer lesion and its accompanying normal squamous epithelium. In the first tumor, a 2-fold or greater up-regulation of expression in the cancer lesion, as compared with its adjacent normal squamous epithelium, was observed for 796 genes (fold-change range: 2–9.9) was observed. In the second tumor, up-regulation was observed in 1,230 genes (fold-change range: 2–2069) and down-regulation was found for 1,360 genes (fold-change range: 2–1.18).

Upon comparing the 2 sets of array results, we found expression of 32 genes to be apparently similarly altered in both the tumors, 8 were commonly up-regulated and 24 were commonly down-regulated (Table I). From this list of 32 potential cervical cancer markers, we chose 6 genes (S100A2, GPC4, p72, IGFBP-5, TRIM2 and NAB2; Table II), based on their known ontology annotation functions, for further study.
with that of epithelia. The mRNA expression ratios of the 6 genes compared mas of the nonkeratinizing-type and 10 normal cervix squamous confirm the microarray results in 14 new squamous cell carcino-

Real-time RT-PCR analysis
Real-time RT-PCR analysis of the 6 selected genes was used to confirm the microarray results in 14 new squamous cell carcinomas of the nonkeratinizing-type and 10 normal cervix squamous epithelia. The mRNA expression ratios of the 6 genes compared with that of GAPDH are shown in Figure 1.

Among the 6 microarray-suggested candidate marker genes, by real time RT-PCR analysis we found a significant change (a decrease) only for the insulin-like growth factor binding protein-5 gene (IGFBP-5; p < 0.001 by the Mann–Whitney U test), when the 14 tumor tissues were compared with the larger number of normal squamous epithelia. The other 5 candidate genes did not present with sufficiently consistent differences in their mRNA expression levels between the carcinomas and normal squamous epithelium.

Ligand-blocking study for the anti-IGFBP-5 antibody
To confirm the specificity of the antibody and the identity of the detected antigen, the IGFBP-5 antibody was preincubated with recombinant human IGFBP-5 protein, and then immunohistochemistry was performed on one of normal squamous epithelium, which was strongly positive for IGFBP-5 immunohistochemistry. Using ligand-preincubated antibody, the positive immunostaining completely vanished, whereas un-preincubated antibody showed robust staining of the same case (Fig. 2).

Immunohistochemical staining of IGFBP-5
Immunohistochemistry was performed to investigate the expression of IGFBP-5 protein in 26 cases of cervical squamous cell

**TABLE I – GENES DIFFERENTIALLY EXPRESSED IN CERVICAL SQUAMOUS CELL CARCINOMA, COMPARED WITH COEXISTENT NORMAL SQUAMOUS EPITHELIUM**

| GenBank no. | Known gene symbol | Case 1* | Case 2* |
|-------------|-------------------|--------|--------|
| Up-regulated gene or EST |
| AF061812 | Keratin 16 (KRT16A) | 2.3 | 2.3 |
| J00124 | 50-kDa type-1 epidermal keratin | 4.7 | 1.3 |
| M59830 | MHC class III HSP70-2 (HLA) | 1.0 | 1.5 |
| L78833 | BRCA1, Rho7, Var-t | 1.4 | 1.5 |
| M28439 | Keratin type-16 (exon 8) | 2.2 | 2.5 |
| M87068 | CaAI | 1.1 | 1.6 |
| AA128249 | Z129d09.x1 | 2.9 | 2.9 |
| AI539439 | Te51e07.x1 | 1.3 | 1.6 |

Down-regulated gene or EST |
| AB023169 | KIAA0952 | -1.9 | -1.2 |
| AB028974 | KIAA0517 | -3.6 | -2.6 |
| ACO04080 | PAC clone D01700O19 from 7p15-p21 | -1.0 | -1.6 |
| ACO04131 | Chromosome 16 BAC clone CT9875K-A-69G12 | -1.0 | -2.1 |
| AF008915 | EVIS homolog | -1.1 | -1.4 |
| AF26692 | Frizzled-related protein frpHE | -1.0 | -2.8 |
| AF030107 | Regulator of G protein signaling (RGS13) | -1.6 | -3.9 |
| AF030186 | Glypican 4 (GPC4) | 1.2 | -2.3 |
| AF043906 | T245 | 2.2 | -1.7 |
| AL049974 | DFKZp564B222 (from clone DFKZp564B222) | -1.7 | -1.5 |
| K03000 | Aldehyde dehydrogenase 1 (ALDH1) | -1.1 | -2.5 |
| M73720 | Mast cell carboxypeptidase A (MC-CPA) | -1.0 | -1.0 |
| U59321 | DEAD-box p72 | 1.2 | -1.9 |
| U82319 | Clone YDD19 | 1.7 | -1.3 |
| D37965 | PDGF-receptor-β-like tumor suppressor (PLRTS) | 1.0 | -1.4 |
| M29960 | Steroid receptor TR2-11 | 1.2 | -1.2 |
| M65062 | Insulin-like growth factor binding protein-5 (IGFBP-5) | 1.2 | -2.2 |
| U04636 | Cyclooxygenase-2 (Cox-2) | -1.2 | -2.2 |
| X03473 | Histone H1(0) | -1.4 | -2.0 |
| AB011089 | KIAA0517 | -1.0 | -1.5 |
| ACO02398 | Human DNA from chromosome 19-specific cosmid F25965 | -1.5 | -2.8 |
| AA402332 | Zu48h12.x1 | -2.2 | -1.6 |
| AI561196 | Tq27a01.x1 | -1.1 | -1.1 |
| AL039458 | DFKZp434N0910 s1 | -1.3 | -2.2 |

*Numbers expressed in the index for 2.

**TABLE II – GENES SELECTED FOR REAL-TIME RT-PCR ANALYSIS**

| Locus ID | Known gene symbol and GenBank no. | Function | Reference |
|----------|----------------------------------|----------|-----------|
| Up-regulated gene |
| NM 005978 | S100 calcium binding protein A2 (S100A2) (AI539439) | Cell cycle progression, cell differentiation | 21,22 |
| AACS0787 | DEAD-box p72 (U59321) | Cellular growth, cell division | 23,24 |
| AOAD4730 | Insulin-like growth factor binding protein-5 (IGFBP-5) (M56062) | Regulation of cell growth, signal transduction | 25,26 |
| NM 015271 | Tripartite motif-containing 2 (TRIM2) (AI561196) | Zinc ion binding, cell proliferation | 27,28 |
| NM 005967 | NGFI-A binding protein 2 (NAB2) | Cell proliferation, neurogenesis | 29 |
| NP 0004190 | Glypican 4 (GPC4) (AI561196) | Cell proliferation, morphogenesis | 30,31 |

Real-time RT-PCR analysis
Real-time RT-PCR analysis of the 6 selected genes was used to confirm the microarray results in 14 new squamous cell carcinomas of the nonkeratinizing-type and 10 normal cervix squamous epithelia. The mRNA expression ratios of the 6 genes compared with that of GAPDH are shown in Figure 1.

Among the 6 microarray-suggested candidate marker genes, by real time RT-PCR analysis we found a significant change (a decrease) only for the insulin-like growth factor binding protein-5 gene (IGFBP-5; p < 0.001 by the Mann–Whitney U test), when the 14 tumor tissues were compared with the larger number of normal squamous epithelia. The other 5 candidate genes did not present with sufficiently consistent differences in their mRNA expression levels between the carcinomas and normal squamous epithelium.
FIGURE 1 – Real-time RT-PCR results for 6 selected genes. The ratios of mRNA expressions of 6 selected genes (S100A2, GPC4, p72, IGFBP-5, TRIM2 and NAB2), relative to GAPDH, analyzed by real-time RT-PCR of 24 cervical tissues, including 14 cases of nonkeratinizing-type cervical squamous cell carcinomas (carcinoma) and 10 cases of normal squamous epithelium (normal cervix), are shown. A significant decrease of IGFBP-5 mRNA was detected in the squamous cell carcinomas, compared with normal squamous epithelium (*; \( p < 0.001 \), by the Mann–Whitney U test). The other 5 candidate genes identified by microarray did not present with consistent differences in mRNA expression levels between the carcinomas and the normal cervical squamous epithelia in this broader set of tumors. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
carcinoma (7 cases of keratinizing-type, 19 cases of nonkeratinizing-type) and 30 cases of normal squamous epithelium, a total of 56 cervical tissues.

In the normal squamous epithelium, all 30 cases demonstrated moderately to strongly positive IGFBP-5 staining of the cytoplasm and the cell membrane, with especially strong staining of the keratinizing epithelium region (Figs. 3a and 3b). Many of the nuclei, especially in the basal cell region and in the maturing squamous cell layer, were also strongly stained with anti-IGFBP-5. Seventy percent (21/30) of normal epithelia demonstrated strong (+) staining and the remaining 9 cases (30%) had positive (+) staining. In contrast to normal squamous epithelium, only 1 (5%) of

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FIGURE 2 – Ligand-blocking study for the anti-IGFBP-5 antibody. Specimens of normal squamous epithelium. Strong cytoplasmic, nuclear and membranous staining with anti-IGFBP-5 antibody was blocked by preincubation with ligand. (a) Staining with normal anti-IGFBP-5 antibody. Robust staining (brown color) in squamous epithelium. (b) Staining with ligand-preincubated anti-IGFBP-5 antibody. The positive staining completely vanished. Original magnification ×100.

FIGURE 3 – Immunohistochemical detection of IGFBP-5 on normal squamous epithelium and cervical cancer. Strong cytoplasmic, nuclear and membranous expression of IGFBP-5 was detected (brown color) in the basal cell and keratinizing-layer of normal squamous epithelium, but was reduced or absent in many squamous cell carcinomas. (a, b) Normal squamous epithelium. Strong (+++) expression of IGFBP-5 in the keratinizing layer. (c, d) Squamous cell carcinoma, nonkeratinizing-type. No (−) expression of IGFBP-5 in the cancerous cells. (a, c) Original magnification ×40; (b, d) Original magnification ×100.
the 19 nonkeratinizing-type cervical squamous cell carcinomas and 1 (14%) of the 7 keratinizing-type cervical squamous cell carcinomas presented strong (+ +) expression of the IGFBP-5 protein. Thirteen (68%) of the 19 nonkeratinizing-type cervical squamous cell carcinoma and 4 (57%) of the 7 keratinizing-type cervical squamous cell carcinoma were completely negative for IGFBP-5 staining (Figs. 3c, 3d and 5). Overall, 65% (17/26) of the SCC tumors had complete loss of IGFBP-5 protein expression, with only 8% (2/26) of the SCC retaining normal strong expression. The difference in staining patterns between a normal cervical squamous epithelium and both the nonkeratinizing and keratinizing-types of cervical squamous cell carcinomas was significant (p < 0.001 for both types by the χ² test).

We next investigated to see at what stage of premalignant lesion of cervical squamous epithelium the expression of IGFBP-5 protein decreased. The normal pattern of strong (+ +) IGFBP-5 stain-
ing was still present in 19 (83%) of 23 squamous metaplasias, and in 15 (75%) of 20 CIN1s and 19 (70%) of 27 CIN2s (Figs. 4a and 5). However, only 5 (24%) of 21 CIN3s showed strong (+ +) staining and 4 (19%) of 21 CIN3s were negative for IGFBP-5 staining (Figs. 4b and 5). The differences in the IGFBP-5 staining scores was significant (p = 0.001) between normal squamous epithelium and CIN 3, whereas the staining score of squamous metaplasia, CIN 1 and CIN 2 were not significantly different compared with the IGFBP-5 staining score of normal squamous epithelium (Fig. 5).

Discussion

In the present study we have analyzed the mRNA expression profiles of 2 individual cases of high-risk-HPV-associated cervical squamous cell carcinomas using the cDNA microarray method and we have compared the expression profile of each tumor with its adjacent normal squamous epithelium from the same patient. We detected expression of approximately 12,000 different genes in each tumor specimen. Similar investigations of other tumor types using this same microarray chip found a comparable number of expressed genes. Out of an estimated possible 24,000 protein coding genes, approximately 16,000–20,000 genes were expressed in leiomyomas and 7,000–17,000 in ovarian cancers. The 12,000 genes detected in each of our specimens thus seem to be an appropriate number.

We found only 8 up-regulated and 24 down-regulated genes common to both SCC tumors. The number of genes found to be ‘commonly altered’ will depend greatly on the number of tumor cases analyzed, as there are often multiple pathways to the same malignant state. It has been suggested that 8 or more individual tumor samples should be included in an analysis to confidently detect tumor-specific commonly-altered-expression genes. With a larger sample size differences in gene expression between the combined groups of carcinomas and normal squamous epithelium can be determined more precisely using statistical analysis tools such as the Student’s t-test.

By using this rigorous but expensive method we were only able to economically analyze 2 cases; however, we were still able to find 32 separate candidate marker genes apparently similarly altered in both tumors to investigate further. However, it is noted that the fold-change found by microarray analysis does not always accurately reflect the absolute up- or down-regulation of gene expression levels. cDNA microarray analysis is known to be a less precise or reproducible method for evaluation of expression levels of mRNA than the PCR-based methods such as real-time PCR, iAFLP or ATAC-PCR, especially for the thousands of genes with relatively low levels of expression. Thus, in our study we performed a real-time RT-PCR analysis for quantitative validation of a sub-set of the candidate genes detected by the cDNA microarray analysis.

In a recent microarray report, Santin et al. examined 5 specimens of cervical squamous cell carcinomas, 5 adenocarcinomas and 1 adenosquamous carcinoma, and compared them with 4 cases of normal squamous epithelium for the expression of 14,500 known genes. All 11 carcinomas were HPV-16 and/or HPV-18 positive. They reported 240 up-regulated and 265 down-regulated genes. As with our microarray results, they also found aldehyde dehydrogenase-1 (ALDH1) to be a down-regulated gene. However, their microarray found the keratin-16 gene to be a down-regulated gene, whereas we found it to be up-regulated. Surprisingly, no other genes found in their study matched any of those found commonly altered in our study.

There has been another reported cDNA microarray investigation involving 600 cancer-related genes in cytology specimens of 3 invasive cervical cancers. These 3 cases were likewise HPV-16 and/or HPV-18 positive. They selected for 3-fold up- or down-regulated genes, when compared with 3 normal squamous cell cytology specimens. They detected 29 up-regulated and 7 down-regulated genes. Interestingly, they also found IGFBP-5 to be a commonly down-regulated gene at the mRNA level, although this was the only commonly altered gene consistent with our own study. By comparison between these 2 other reports, Hudelist et al. found only 2 commonly altered genes, cyclin-dependent kinase inhibitor 2A and E2F transcription factor 1, listed in both as up-regulated, but we did not find these to be up-regulated in our 2 carcinomas.

There are numerous factors which affect the sensitivity, accuracy and reproducibility of microarray analysis of human surgical specimens, such as the selection and number of specimens, their HPV-infection status, sample collection methods, accuracy of microdissection isolation, presence of nonmalignant supporting cells, RNA isolation, the kind of microarray, target preparation, microarray hybridization, and method of chip analysis. In microarray analysis, serious limitations may occur in the reliability of the data generated from low significance genes and the elimination of unreliable signal intensities near the optimal thresholds is often needed. Therefore, we, like others, have found that the validation of a microarray-suggested altered gene expression with independent quantitative methods, such as real-time RT-PCR analysis and immunohistochemistry, is an absolute necessity.

The production of mRNA splice-variants is another technical problem in the application of cDNA microarray analysis. The signal detection by microarray is based on the hybridization to oligonucleotide probes, and most microarray designs are not intended to detect the changes in a single splicing variant, and thus have this limitation in the measurement of tissue-specific splice variants. However, alternative splicing has an important role in the regulation of different tissues and approximately 30–60% of human genes undergo alternative splicing. For the estimation and prediction of splice variants from cDNA microarray data, Hu et al. reported a new algorithm to normalize and compare the variants across different tissues. However, cDNA microarrays designed for specific splicing variants are not generally used at present. Thus, alternative splicing of mRNA may be another possibility as to why the alteration of mRNA expression detected in microarray analysis was confirmed in only IGFBP-5 by real-time RT-PCR, out of the 6 genes projected by cDNA microarray analysis. However, we do not know the precise location of the oligo-probes for the genes and the ESTs on the Affymetrix Gene Chip U95 arrays relative to the location of the primers we used for real-time RT-PCR analysis.

Through rigorous real-time RT-PCR analysis and immunohistochemical staining to test our microarray findings, we were able to confirm that at least for IGFBP-5 a significantly reduced expression of the protein paralleled the gene’s down-regulated mRNA in the majority of cervical squamous cell carcinomas, suggesting that this is a frequently used pathway associated with the genesis of this tumor type. We also show that it is observed even in the pre-malignant intramucosal squamous lesion.

The ever-growing IGFBP family is composed of at least 7 highly conserved members, IGFBP-1 to -7, 25–40 kDa in size. Specific IGFBPs are secreted by many cell types into the extracellular milieu where they can bind to insulin-like growth factors (IGF)-I or IL. The major component of IGF-binding protein in blood is IGFBP-3.

In humans, the IGFBP-5 gene spans 33 kb on chromosome 2. The major sites of IGFBP-5 expression in normal human tissues include the testis, bone trabecular meshwork, lung, and important for this study, the ovary, uterus and placenta. IGFBP-5 plays a significant role in the normal regulation of organ function, including the development of the central nervous system, involucration of the mammary gland, and bone physiology. It has been demonstrated that the urotropic agent estradiol suppresses expression of IGFBP-5 gene through increased IGF-1 production in the rat uterus. This suggests strong links between the estrogenic and IGF/IGFBP-5 growth regulating pathways in estrogen-responsive tissues.

IGFBP-5 exerts biological activities not only in the presence of, but also in the absence of IGFs, indicating the existence of IGFR-
dependent signaling pathways for IGFBP-5. Both IGFBP-3 and IGFBP-5 share a common nuclear transport pathway, suggesting that they have the potential for nuclear transport and direct interaction with DNA as transcriptional regulatory co-factors. The nuclear localization of IGFBP-5, which we observed in our immunohistochemistry, may thus reflect such a nuclear role of IGFBP-5 in cervical carcinogenesis.

IGFBP-5 appears to play a suppressive role against the cell proliferation of some kinds of tumors. Down-regulation of IGFBP-5 has been found to be involved in the neoplastic transformation of oral keratinocytes and in renal clear cell carcinogenesis. On the other hand, IGFBP-5 acts in concert with IGF-II in inducing cell proliferation in other tumor types, for example, the over-expression of IGFBP-5 is a common feature of neuroblastoma cell lines.

The majority of advanced cervical intraepithelial neoplasia (CIN II/III) and SCC are actively infected with HPV and have an up-regulated epidermal growth factor receptor (EGF-R). In clinical studies, HPV infection and up-regulation of the EGF-R in the cervical epithelium are linked to the up-regulation of IGF-II serum levels and decreased IGFBP-3 serum levels.

The E7 protein of HPV-16 has been shown to directly inactivate the IGFBP-3 protein, the first suggestion of a possible direct involvement of the IGFBP family in HPV-infection-mediated cervical carcinogenesis. Subsequently it has been shown that clinical staging of cervical cancers is significantly related to the expression of the IGFBP-1 and -2 proteins. IGFBP-1, -2 and -3 mRNAs are detected in 6%, 91% and 97%, of cervical tumors, and their proteins in 44%, 19%, and 84% of cases.

A study of IGFBP-3 gene expression in primary cervical epithelial cells during a time course after retroviral transduction with either low-risk or high-risk E6/E7 genes found that, at late passages after high-risk HPV-E6/E7 expression, transforming cervical cells exhibited dramatic over-expression of IGFBP-3 mRNA and of both secreted and intracellular IGFBP-3 protein, with the mRNA levels increasing approximately 85-fold. In situ hybridization studies of cervical biopsies revealed high levels of IGFBP-3 mRNA expression in high-grade squamous intraepithelial neoplasia but not in normal cervical epithelium.

In seeming contrast to what has been found with IGFBP-3, it was reported that epidermal growth factor (EGF)-induced mitogenesis in cervical cancer was abrogated in a dose-dependent manner by treatment with IGFBP-5, which reduced levels of both the EGF-R and autocrine-expressed IGF-II. It has also been demonstrated that retinoic acid (RA)-induced expression of IGFBP-5 in cervical cancer cell lines resulted in growth inhibition, a result duplicated by adding exogenous IGFBP-5, indicating that normal IGFBP-5 levels may be suppressive of cervical epithelial growth. Down-regulation of the IGF-1 receptor by antisense RNA reversed the transformed phenotype of a cervical cancer cell line, and up-regulation of IGF-II is detected in clinical biopsy samples of cervical cancers and in the sera from both CIN and cervical cancer patients. IGF-I and II growth promoting signaling, and the endogenous IGFBP-5, which is growth-suppressive by sequestering them, may thus play an important role in the balance of proliferation and invasion of cervical cancer cells.

Unlike for IGFBP-3, the direct effects of HPV-coded proteins on IGFBP-5 signaling are not known. As mentioned, IGFBP-5 inhibits the growth of cultured cervical cancer, especially in HPV-negative cell lines. This may suggest that some of the products of HPV have antagonistic effects to the IGFBP-5 protein’s functions; however, it is also now clear from our results that at least some of the down-regulation of IGFBP-5 protein occurs at the level of mRNA, which is different from the direct inactivation of IGFBP-3 protein by HPV E7. Our current report may suggest the latter hypothesis, that an HPV-coded protein may down-regulate the transcription of IGFBP-5 mRNA. This interaction should be the basis for further investigations.

In our current study, we find that both the mRNA and the protein of IGFBP-5 were significantly decreased in the clinical samples of SCC compared with normal cervical squamous epithelium (p < 0.001, p < 0.001, respectively). This seems to make good biological sense, since IGFBP-5 has a suppressive effect on cervical tumor growth. However, it is still to be determined in further studies how much of the down-regulation of IGFBP-5 protein expression in cervical cancers is due to the myriad of potential regulatory pathways such as: alterations in IGFBP-5 transcription regulated by the estrogen-mediated pathway, the increased IGF-ligand levels, the other IGFBPs or IGFBP-associated binding proteins, the IGFBP-5-specific extracellular proteases or intracellular-targeted degradation, or by direct inactivation of IGFBP-5 by HPV proteins.

Changes in expression of IGFBP-5 protein have not to our knowledge been previously reported in the premalignant CIN lesions of the uterine cervix. Here, we demonstrate that the loss of IGFBP-5 protein is detected not only in the advanced stages of squamous cell carcinomas, but it has already occurred at the preneoplastic CIN3 stage. However, CIN1, CIN2 and squamous metaplasia expressed IGFBP-5 at nearly the same high levels seen in normal cervical squamous epithelium.

In this study we have shown that the consistently decreased expression of IGFBP-5 mRNA and protein in cervical squamous epithelium is strongly correlated with progression to a squamous cell carcinoma, probably as a moderately early preneoplastic event (CIN3) in squamous carcinogenesis, which suggests a role for IGFBP-5 as a marker of cancer progression in cervical epithelium and may hold a potential pathway for treatment.

References
1. Jones SB. Cancer in the developing world: a call to action. BMJ 1999;319:505–8.
2. Baseman JG, Koutsky LA. The epidemiology of human papillomavirus infections. J Clin Virol 2005;32 (Suppl 1):S16–S24.
3. Pecorelli S, Favalli G, Zigliani L, Oodicino F. Cancer in women. Int J Gynecol Obstet 2003;82:369–79.
4. Chen PG, Sung HY, Sawaya GF. Changes in cervical cancer incidence after three decades of screening US women less than 30 years old. Obstet Gynecol 2003;102:765–73.
5. Smith HO, Tiffany MF, Qualiss CR, Key CR. The rising incidence of adenocarcinoma, relative to squamous cell carcinoma of the uterine cervix in the United States—a 24-year population-based study. Gynecol Oncol 2000;78:97–105.
6. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: relative risk associations I: human tumor antigenic types. Obstet Gynecol 1992;92:327–38.
7. Clavel C, Masure M, Bory JP, Putaud I, Mangeonjean C, Lorenzato M, Gabriel R, Quevreux C, Birembaut P. Hybrid Capture II-based human papillomavirus detection, a sensitive test to detect in routine high-grade cervical lesions: a preliminary study on 1518 women. Br J Cancer 1999;80:1306–11.
8. Motoyama S, Laines-Llave CA, Luis Villanueva S, Maruo T. The role of human papilloma virus in the molecular biology of cervical carcinogenesis. Kobe J Med Sci 2004;50:9–19.
9. Bedell MA, Jones KH, Laimins LA. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and rat-1 cells. J Virol 1987;61:3635–40.
10. Phelps WC, Yee CL, Munger K, Howley PM. The human papillomavirus type 16 E7 gene encodes transactivating transfection functions similar to those of adenovirus E1A. Cell 1988;53:539–47.
11. Spitkovsky D, Aengenestfnd F, Brasperning J, von Knebel Doeberitz K. Regulation of cervical cancer cell lines by the papillomavirus E6 oncogene. Oncogene 1996;13:1027–35.
12. Storey A, Pin D, Murray A, Osborn K, Banks L, Crawford L. Comparison of the in vitro transforming activities of human papillomavirus types. EMBO J 1988;7:1815–20.
Ward P, Coleman DV, Malcolm AD. Regulatory mechanisms of the DHX9 gene ranking system. Nucleic Acids Res 2001;29:156–8.

Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler A, et al. Gene structure-based splice variant deconvolution using a microarray platform. Bioinformatics 2003;19 (Suppl 1):i315–i322.

Santin AD, Zhan F, Bignotti E, Siegel ER, Cane S, Bellone S, Palacci L. Essential roles of IGF-binding proteins in breast cancer. Eur J Cancer 2005;41:1515–27.

Kawamoto S, Ohnishi T, Kita H, Chisaka O, Okubo K. Expression profiling of primary HPV-16- and HPV-18-infected early stage cervical carcinomas and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy. Virology 2005;331:269–91.

Napoli C, Lemann LO, Sica V, Lemann A, Tajana G, de Nigris F. Microarray analysis: a novel research tool for cardiovascular scientists and physicians. Heart 2003;89:597–604.

Ueda Y, Enomoto T, Miyatake H, Kato Y, Kanoa J, Uera AJ, Dickson KA, McDougall KR, Murata Y. Monoclonal expansion with integration of high-risk-type human papillomaviruses is an initial step for cervical carcinogenesis: association of clonal status and human papillomavirus infection with clinical outcome in cervical intraepithelial neoplasia. Lab Invest 2003;83:1517–27.

Skubitz KM, Skubitz AP. Differential gene expression in uterine leiomyoma. J Lab Clin Med 2003;141:297–308.

Kawasaki S, Ohnishi T, Kita H, Chisaka O, Okubo K. Expression profiling of primary HPV-16- and HPV-18-infected early stage cervical carcinomas and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy. Virology 2005;331:269–91.

Hodak J, Czerwienka-Singer C, Pochopinska K, Kubista E, Manavi M. cDNA array analysis of cytobrush-collected normal and malignant cervical epithelial cells: a feasibility study. Cancer Genet Cytofgenet 2005;158:35–42.

Wang H, Hubbell E, Hu JS, Mei G, Cline M, Lu G, Clark T, Siani-Rose MA, Ares M, Kulp DC, Haussler D. Gene structure-based splice variant deconvolution using a microarray platform. Bioinformatics 2003;19 (Suppl 1):i315–i322.

Saigo S, Matoba R, Kato K. Adapter-tagged competitive PCR (ATAC-PCR)—a high-throughput quantitative PCR method for microarray validation. Methods 2003;13:326–31.
members of the IGF-axis in clear cell renal cell carcinoma. Int J Oncol 2005;26:923–31.

63. Cesi V, Vitali R, Tanno B, Giuffrida ML, Sesti F, Mancini C, Raschella G. Insulin-like growth factor binding protein 5: contribution to growth and differentiation of neuroblastoma cells. Ann N Y Acad Sci 2004;1028:59–68.

64. Mathur SP, Mathur RS, Young RC. Cervical epidermal growth factor-receptor (EGF-R) and serum insulin-like growth factor II (IGF-II) levels are potential markers for cervical cancer. Am J Reprod Immunol 2000;44:222–30.

65. Mathur SP, Mathur RS, Rust PF, Young RC. Human papilloma virus (HPV)-E6/E7 and epidermal growth factor receptor (EGF-R) protein levels in cervical cancer and cervical intraepithelial neoplasia (CIN). Am J Reprod Immunol 2001;46:280–7.

66. Mathur SP, Landen CP, Datta SM, Hoffman MC, Mathur RS, Young RC. Insulin-like growth factor II in gynecological cancers: a preliminary study. Am J Reprod Immunol 2003;49:113–19.

67. Mathur SP, Mathur RS, Gray EA, Lane D, Underwood PG, Kohler M, Creasman WT. Serum vascular endothelial growth factor C (VEGF-C) as a specific biomarker for advanced cervical cancer: relationship to insulin-like growth factor II (IGF-II), IGF binding protein 3 (IGF-BP3) and VEGF-B. Gynecol Oncol 2005;98:467–83.

68. Mathur SP, Mathur RS, Underwood PB, Kohler MF, Creasman WT. Circulating levels of insulin-like growth factor-II and IGF-binding protein 3 in cervical cancer. Gynecol Oncol 2003;91:486–93.

69. Mannhardt B, Weinzimmer SA, Wagner M, Fiedler M, Cohen P, Jansen-Durr P, Zwierschke W. Human papillomavirus type 16 E7 onco-protein binds and inactivates growth-inhibitory insulin-like growth factor binding protein 3. Mol Cell Biol 2000;20:6483–95.

70. Steller MA, Delgado CH, Zou Z. Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells. Proc Natl Acad Sci USA 1995;92:11970–4.

71. Berger AJ, Baege A, Guillemette T, Deeds J, Meyer R, Disbrow G, Schlegel R. Insulin-like growth factor-binding protein 3 expression increases during immortalization of cervical keratinocytes by human papillomavirus type 16 E6 and E7 proteins. Am J Pathol 2002;161:603–10.

72. Hirano S, Ito N, Takahashi S, Tamaya T. Clinical implications of insulin-like growth factors through the presence of their binding proteins and receptors expressed in gynecological cancers. Eur J Gynaecol Oncol 2004;25:187–91.

73. Higo H, Duan C, Clemmons DR, Herman B. Retinoic acid inhibits cell growth in HPV negative cervical carcinoma cells by induction of insulin-like growth factor binding protein-5 (IGFBP-5) secretion. Biochem Biophys Res Commun 1997;239:706–9.

74. Nakamura K, Hongo A, Kodama J, Miyagi Y, Yoshinouti M, Kudo T. Down-regulation of the insulin-like growth factor I receptor by antisense RNA can reverse the transformed phenotype of human cervical cancer cell lines. Cancer Res 2000;60:760–5.

75. Shen MR, Lin AC, Hsu YM, Chang TJ, Tang MJ, Alper SL, Ellory JC, Chou CY. Insulin-like growth factor I stimulate KCL cotransport, which is necessary for invasion and proliferation of cervical cancer and ovarian cancer cells. J Biol Chem 2004;279:40017–25.