Changes in Cervical Keratinocyte Gene Expression Associated with Integration of Human Papillomavirus 16

William Alazawi, Mark Pett, Barbara Arch, Laurie Scott, Tom Freeman, Margaret A. Stanley, and Nicholas Coleman

Medical Research Council Cancer Cell Unit, MRC/Hutchison Research Centre, Cambridge CB2 2XZ [W. A., N. C.]; Department of Pathology, University of Cambridge, Cambridge CB2 1QG [M. P., M. A. S., N. C.]; Institute of Public Health, Cambridge CB2 2SR [B. A.]; and Medical Research Council Human Genome Mapping Resource Centre, Cambridge CB10 1SB [L. S., T. F.], United Kingdom

ABSTRACT

Episomal integration is a critical event in human papillomavirus (HPV)-related oncogenesis, although little information is currently available concerning the effect of integration on the host transcriptome. We have used expression microarrays to investigate the effect of integration of HPV16 on gene expression in cervical keratinocytes, using the unique cell line model W12. W12 was generated from a cervical low-grade squamous intraepithelial lesion “naturally” infected with HPV16 and at low passage contains approximately 100 HPV16 episomes/cell. With passage in vitro, integration of viral episomes is associated with the development of phenotypic and genomic abnormalities resembling those seen in cervical neoplastic progression in vivo. We have used the Affymetrix U95A oligonucleotide array that contains probes for 12,600 human transcripts and have identified 85 genes from a range of host cell pathways that show changes in expression levels after integration of HPV16. Whereas some of the genes have previously been implicated in HPV-related oncogenesis in vivo, we have also identified a range of genes not previously described as being involved in cervical neoplastic progression. Interestingly, integration is associated with up-regulation of numerous IFN-responsive genes, in comparison with a baseline of episomically infected cells. These genes include p48, a component of the primary regulator of the IFN response pathway, IFN-stimulated gene factor 3. The physical state of high-risk HPV may substantially influence the response to IFN in infected keratinocytes.

INTRODUCTION

SCC 4 of the cervix evolves through well-defined noninvasive stages, which may be classified using a two-tier system as LG-SIL and HG-SIL. Cervical squamous neoplasia is strongly associated with infection with HPV (1–3), and integration of high-risk HPV—such as types 16, 18, 31, and 33—is associated with malignant progression. Whereas in premalignant cervical lesions the HPV genome is typically maintained in its episomal form (4), integration has been shown in over 80% of HPV16-positive SCCs (4) and in 100% of HPV18-positive SCCs (5, 6).

HPV integration is usually accompanied by disruption of the viral E1 and E2 genes, with release of repression of the transforming genes E6 and E7 (7). It is likely that HPV infection and integration cooperate with changes in the host genome and transcriptome in the multistep process of cervical carcinogenesis (1). However, little information is currently available concerning changes in host gene expression that are associated with integration of HPV in cervical keratinocytes.

Two recent investigations have used cDNA microarrays to examine changes in gene expression in human foreskin keratinocytes after transfection with high-risk HPVs (8) and low-risk HPV11 (9). In both studies, the virus remained in the episomal state. Interestingly, 11 IFN-responsive genes were down-regulated after transfection with HPV31 episomes (8). In contrast, 2 of these 11 genes [IFN-induced p27 (11.5 kDa) and IFN-induced 56 kDa] were reported to show increased expression levels (and the other 9 no change) after transfection with HPV11 episomes (9). A third study used cDNA microarrays to demonstrate reduced expression of at least 18 IFN-responsive genes after infection of cervical keratinocytes with retroviruses encoding high-risk HPV16 E6 and E7 and in vitro induction of growth arrest and terminal differentiation (10).

In the present study, we have investigated changes in host gene expression in cervical keratinocytes that are associated with integration of high-risk HPV16, the HPV type most commonly found in cervical carcinomas (1–3). We have used as a model the W12 cell line, which was generated from a cervical LG-SIL and, thus, arose after “natural” cervical infection with HPV16 in vivo (11). At early passages, W12 is diploid and contains approximately 100 HPV16 episomes/cell (11). Our group has shown in a parallel study 5 that episomal integration (which typically occurs between passages 10 and 22) is accompanied by an increase in the grade of lesion that W12 recapitulates in organotypic culture, enhanced colony-forming efficiency and growth rate, and a stepwise accumulation of host cytogenetic abnormalities that are similar to those seen in cervical neoplasia in vivo (12, 13). W12 is, therefore, a valuable and unique reagent, being a naturally infected cervical keratinocyte cell line that represents an accurate model of progression of HPV16-associated cervical neoplasia.

We have used oligonucleotide microarrays to investigate changes in expression of 12,600 human transcripts and expressed sequence tags associated with integration of HPV16 in W12. We have examined three passages of W12 in which we have characterized the physical state of the HPV16 genome by Southern hybridization. In a separate study, all passes of W12 tested were shown by chromosome painting to be polyclonal. 5 The passages analyzed were:

(a) W12p10, which contains episomal HPV16 only and is near diploid. We used W12p10 as a baseline for analysis of gene expression changes in higher passages.

(b) W12p22, which contains integrated HPV16 only and is near tetraploid. W12p22 shows cytogenetic abnormalities that are not seen in W12p10 or W12p14, most notably a high-level gain of chromosome 5p, which is present in approximately 50% of metaphases of W12p22 and becomes more frequent in later passages. 6 Comparison of the transcriptome between W12p22 and W12p10 would enable us

Received 4/4/02; accepted 10/4/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Funded by the Medical Research Council.

2 Joint first authors.

3 To whom requests for reprints should be addressed, at Medical Research Council Cancer Cell Unit, MRC/Hutchison Research Centre, Hills Road, Cambridge CB2 2XZ, United Kingdom. Phone: 44-1223-763285; Fax: 44-1223-763284; E-mail: nc109@cam.ac.uk.

4 The abbreviations used are: SCC, squamous cell carcinoma; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, EGF receptor, HG-SIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; IGF, insulin-like growth factor; LG-SIL, low-grade SIL.

5 Personal communications; 19th International Papillomavirus Conference, 2001, Florianopolis, Brazil: Alazawi et al., Changes in the host cell genome and transcriptome associated with integration of HPV16 in cervical keratinocytes; and Dowen et al., Gain of chromosome arm 5p: a recurrent late cytogenetic event in HPV16 and HPV18 expressing cell lines.
to identify changes in cervical keratinocyte gene expression resulting from the complete process of integration.

(c) W12p14, which contains mixed episomal and integrated HPV16 and is near tetraploid. In the present study, we show that there is an approximately 70% reduction in HPV16 episomal signal by Southern blot in W12p14 compared with W12p10. Comparison of gene expression between W12p14 and W12p10 would allow us to examine effects of early disruption of the HPV16 episome (which we also show in the present study to result in increased levels of HPV16 E7 protein and loss of HPV16 E2 mRNA) and the critical switch in the host genome from diploidy to tetraploidy. Comparison of gene expression between W12p22 and W12p14 would enable us to examine effects of complete loss of HPV16 episomes and the associated cytogenetic abnormalities (e.g., high-level gain of chromosome 5p).

All passages of W12 analyzed were grown in monolayer tissue culture, which selects for cells with a proliferative advantage and limits differentiation (14). These conditions enable W12 to serve as a model of HPV16 infection of basal cervical squamous cells. This is of particular importance, because derepression of viral E7 and E6 genes in the basal layers of the cervix, as occurs after episomal integration, is an important early event in cervical carcinogenesis (15) (16).

We have identified genes from several host cellular pathways that show changes in expression levels during the process of integration of HPV16 episomes in cervical keratinocytes. Several of the changes have been demonstrated previously to occur in cervical neoplasia in vivo, further confirming the validity of W12 as a model system for cervical oncogenesis. We have also identified numerous other, previously unreported changes in gene expression that may also contribute to cervical oncogenesis in vivo.

MATERIALS AND METHODS

Monolayer Cell Culture and Extraction of Nucleic Acids. W12 keratinocytes were grown in Glasgow’s modified Eagle’s medium supplemented with FCS and EGF, in the presence of irradiated murine 3T3 J2 fibroblast feeder cells, as described previously (11, 17). Culture conditions were the same for all passages of W12 examined. Normal ectocervical cells were obtained after hysterectomy for disease unrelated to the cervix and were cultured as described previously (17). At approximately 80% confluence, total RNA and DNA were extracted from the keratinocytes using the TRIzol extraction kit (Life Technologies, Inc., Grand Island, NY). All samples of W12 investigated were polyclonal.3 In all cases, RNA and DNA were obtained from at least three independently grown tissue culture flasks.

Southern Analysis of the Physical State of HPV16. DNA (10 μg) from W12 (at passes 10, 14, 15, 22, 34, 44, and 55) and 1 μg of DNA from the cervical SCC cell line CaSkI (18) were digested with BamHI or HindIII and electrophoresed through 0.8% agarose. For lanes loaded with 10 μg of DNA, we used copy number controls representing 1, 10, and 100 copies of linearized HPV16/diploid cell. After partial depurination, denaturation, and neutralization, DNA was transferred to Hybond-N nylon membrane (Amersham, Little Chalfont, United Kingdom). Prehybridization and hybridization of 32P-labeled HPV16 probe were performed at 65°C using Rapid-Hyb buffer (Amersham) for 1 h and 2.5 h, respectively. The probe for hybridization was prepared by excision and purification of full-length HPV16 from the pSPHPV16 plasmid, followed by labeling with α-32P-dCTP using random priming. After hybridization membranes were washed and exposed to autoradiographic film (Amer- sham) at −70°C using intensifying screens.

Western Blotting for HPV16 E7 Protein. Total protein was extracted from at least three separate flasks of W12 (at passes 10, 14, 23, 35, 43, and 56), the cervical SCC cell line CaSkI (18), and normal ectocervical keratinocytes at approximately 80% confluence. The protein extraction buffer contained 250 mM NaCl, 0.1% NP40, 50 mM HEPES, and one protease inhibitor tablet (Roche, Basel, Switzerland) per 25 ml. Extracts were stored in 10–20% glycerol at −70°C. Twenty micrograms of total protein was run on a 12.5% polyacrylamide gel, blotted, and analyzed by hybridization of a sheep poly-}

clonal antibody against HPV16 E7 (Ref. 19; a kind gift from Cantab Pharmaceuticals, Cambridge, United Kingdom).

RT-PCR for HPV16 E2 mRNA. Total RNA was extracted from at least three separate flasks of W12 (at passes 10, 15, 24, and 55), the cervical SCC cell lines CaSkI (18) and SiHa (20), and normal ectocervical keratinocytes. As a positive control, we used the plasmid pSPHPV16 (11), which contains the full-length HPV16 genome. The RNA was reverse transcribed using a SuperScript reverse transcription kit (Life Technologies, Inc.), including a reverse transcriptase-negative control for each sample. The resultant samples were amplified using primers for E2 (forward, 5′-TGCAGTGATCCGT- GAGACTCTTTGCACG-3′; reverse, 5′-TGCAGTGATCTCTCATA- GACATAATCCG-3′), and the products were run on a 1% agarose gel. The expected product size was 1139 bp.

Hybridization to Affymetrix Arrays. Biotin-labeled target cRNA was generated from total RNA from W12p10, W12p14, and W12p22. We generated two assay replicates from total RNA from W12p10 and W12p14 and three assay replicates from total RNA from W12p22. Generation of cDNA, biotin labeling, and fragmentation of cRNA were performed according to Affymetrix (Santa Clara, CA) protocols. In brief, double-stranded cDNA was generated using the T7 (-dT)24 primer 5′-GGCCAGTGAATTGATACGTAC- TATAGGAGGCGG-3′ and Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). Biotin-labeled cRNA was generated from this using the Enzo BioArray HighYield RNA Transcription labeling kit (Enzo, New York, NY).

cRNA from each assay replicate of each passage of W12 was hybridized to an Affymetrix human U95A oligonucleotide array slide for 16 h at 45°C in a hybridization mixture (Affymetrix). Each array contained probes for 12,600 transcripts. After hybridization the slides were washed and stained using fluidics protocols described in the Affymetrix manual.

Analysis of Microarray Data. Gene expression was assessed using algorithms designed by Affymetrix in the Microarray Suite software package (21). For each sample, genes were classified as either “present,” “marginal,” or “absent,” with a numerical indication of average intensity difference between the perfect match and central-base mismatch features. A global normalization algorithm and linear regression scaling factor were applied to normalize data from different hybridizations. Multiple parameters (21) were used to generate numerical fold changes reflecting the relative abundance of each transcript in each pair of samples. Changes in expression were also assigned into one of five discrete categories: “decrease,” “marginal decrease,” “no change,” “marginal increase,” and “increase.”

Affymetrix data were analyzed further using Microarray Suite Empirical and Statistical expression algorithms (Affymetrix) and ChipMining Tool soft-}

ware (Gareth Denyer, Sydney, Australia). When comparing gene expression between passages of W12, we excluded genes from further analysis if they were in the “no change,” “marginal decrease,” or “marginal increase” categories and if their numerical fold change was between 2 and −2. Each replicate sample was compared with each sample from the other passage, and only gene expression changes that were identified consistently were analyzed further. These exclusion criteria were similar to those reported by others (22).

Analysis of Gene Distribution. We investigated whether there was overall clustering of the chromosomal locations of genes showing differences in expression levels between passages of W12. We compared the observed distribution of these genes with that expected under the null hypothesis that the genes were evenly distributed throughout the genome. Under this hypothesis, the number of genes located on each chromosome arm was determined from mapping information provided by Affymetrix. A multinomial distribution was used and significance was assessed using the χ2 goodness-of-fit test with a 5% significance level.

When evidence of clustering was detected, a post hoc analysis was carried out to identify whether particular arms were observed as the location of a significant number of genes. In this analysis, only arms representing the location of three or more genes were investigated. Significance was assessed by comparing observed numbers with those expected under the null hypothesis that the number of genes on an arm were binomially distributed, and that genes were evenly distributed throughout the genome. A 2.5% significance level was used to help correct for problems of multiple testing that may have been incurred.
RESULTS

Southern Analysis of the Physical State of HPV16. For W12p10 BamHI (a HPV16 single cutter) produced a band of 7.9 kb (Fig. 1A), representing full-length HPV16 episomes at approximately 100 copies/cell, consistent with previous observations (11). Digestion with HindIII (a HPV16 noncutter) revealed bands consistent with monomeric episomal HPV16, including low molecular weight supercoiled monomers (Fig. 1B). Also present were high molecular weight (>30 kb) bands suggesting the presence of head-to-tail multimeric episomal DNA, which was subsequently confirmed by Southern analysis after two dimensional gel electrophoresis (data not shown). Thus, W12p10 harbors full-length HPV16 episomes in monomeric and head-to-tail multimeric forms. Analysis of W12 at the higher passages used in this study revealed a rapid reduction in the intensity of bands representing episomal DNA, with approximately 70% loss in W12p14, 90% loss in W12p15, and absence in W12p22 (Fig. 1). There was concurrent emergence of bands representing integrated HPV16 DNA. We observed one band after HindIII digestion and two bands after BamHI digestion, indicating a single host integration site containing a single type of viral integrant with one BamHI site. The integrated genomes were maintained at the same copy number in subsequent passages. The cells containing integrated HPV16 were tetraploid,5 and copy number controls suggested approximately 10 copies of integrated HPV16/cell, consistent with amplification of viral DNA after integration of the HPV16 genome (23).

Expression of HPV16 E7 mRNA. HPV16 E7 RNA was detectable by RT-PCR in W12p10 (episomes only), but was lost during the process of integration in W12p15 and W12p24 (Fig. 3). HPV16 E2 was detectable in the cervical SCC cell line CaSki, in which the full-length early region is retained in the integrated HPV16 but is absent from the cervical SCC cell line SiHa, in which the E2 gene is deleted, and from normal ectocervical keratinocytes (NCx). Each reaction was performed in duplicate, with and without reverse transcriptase (left and right lanes of each pair, respectively). The plasmid pspHPV16 (11), which contains the full-length HPV16 genome, was used as a positive control and we included a no-template negative control.

Expression of HPV16 E7 Protein. HPV16 E7 was detectable by Western blotting in W12 at all passages from W12p10 to W12p56. Episomal integration (which occurs between W12p10 and W12p23) is associated with an increase in levels of detectable HPV16 E7 protein. HPV16 E7 is also detectable in the cervical SCC cell line CaSki, but not in normal ectocervical keratinocytes (NCx).

Microarray Analysis of Host Gene Expression. We analyzed two replicates of labeled RNA from W12p10, two replicates of labeled RNA from W12p14, and three replicates of labeled RNA from W12p22. The mean number of genes called as "marginal" or "present" was 3573 (range, 3480–3667) in W12p10, 4166 (range, 4026–
Fig. 4. Stylized Venn diagram depicting the patterns of changes in gene expression levels during the process of HPV16 integration in W12. The principal sets contain the genes showing differences between different passages of W12. Regions of overlap contain genes common to two or more comparisons. The Table gives the numbers of genes that show increased or decreased expression levels at higher passage, subdivided by set.

<table>
<thead>
<tr>
<th>Set</th>
<th>Increase</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td>F</td>
<td>21</td>
<td>16</td>
</tr>
</tbody>
</table>

E = 12660

W12p22 vs. W12p10

W12p14 vs. W12p10

W12p22 vs. W12p14

DISCUSSION

We have used W12 to study the changes in expression levels of 12,600 transcripts that occur during integration of HPV16 episomes in cervical keratinocytes. W12 was derived from a naturally infected cervical LG-SIL (11) and represents a valuable and unique model for investigations of the natural history of cervical squamous cell neoplasia. During passage of W12, the viral integration that we have demonstrated is associated with morphological and cytogenetic changes similar to those seen in cervical neoplastic progression in vivo. In the present study, we have shown that integration of HPV16 in W12 is associated with loss of expression of E2 mRNA and with increased levels of E7 protein (consistent with loss of E2-mediated repression (25–27). It is likely that these events exert direct or indirect effects on the expression of host genes in W12 cells. W12p10 was, therefore, used as a baseline for comparative analysis of changes in gene expression seen at higher passages.

Analysis of W12 by comparative genomic hybridization has shown that progression during passage is associated with the acquisition of nonrandom copy number imbalances. In particular, between W12p10 and W12p22 we observed evidence of loss of 10p and gain of 10q as a result of the formation of an isochromosome (10q), which we have confirmed by spectral karyotyping (19). This isochromosome is then maintained through to high passage. Our present data showing significant clustering of genes up-regulated after HPV integration on 10q and of genes down-regulated after HPV integration on 10p is consistent with our comparative genomic hybridization and spectral karyotyping data.

On the other hand, not all genes showing expression differences in the present study correspond to regions of copy number imbalance detectable in W12 by comparative genomic hybridization. Although
such disparities may, in some cases, reflect the limited resolution of comparative genomic hybridization using chromosome targets, epigenetic factors are also likely to underlie the altered expression of some of the genes that we have identified using microarrays. Despite the high level gain of chromosome 5p seen by us in W12p14 and W12p22 but not W12p10,4 there was no change in the levels of expression of genes on chromosome 5p between W12p10 and later passages. This is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).

The 85 genes in Table 1 are of potential significance in the biology of cervical squamous cell neoplasia in vivo. Several of the genes have been demonstrated in previous studies to change their expression levels during the development of SCC in the cervix. These changes include increased expression at the protein level of thrombomodulin (29) and the antiapoptotic Bcl-2 family member Mcl-1 (Ref. 30; both in set B). There are also genes for which changes in expression levels have been shown in malignancies at other sites, but not in the cervix, to date, such as acid ceramidase (set D), which is up-regulated at the mRNA level in prostatic adenocarcinoma (31); and Mac2-binding protein (set B), which is induced at the protein level in breast and other adenocarcinomas (32).

Several genes with immunomodulatory function showed altered expression in W12 after integration. This observation is of interest, in view of the apparent switch from local immune quiescence in cervical adenocarcinomas (32). Mcl-1 expression in normal and malignant colonic glands is also decreased in the wild-type Mcl-1 expressing mouse (33), suggesting that normal increase may contribute to local immune quiescence. However, the expression of Mcl-1 in adenocarcinomas is not decreased, and the increased expression of CD58 (set A) is consistent with recent data showing that chromosomal regions amplified in neoplasms may be transcriptionally silent (28).
class IF (set D), and the immunostimulatory molecule Mac 2-binding protein (set B; Ref. 33). We also observed that numerous IFN-responsive genes were up-regulated following integration. These genes include p48 (set C), a component of the primary regulator of the IFN response pathway, IFN-stimulated gene factor 3. These data suggest that the physical state of high-risk HPV may substantially influence the response to IFN in infected keratinocytes.

Several of the changes in gene expression that we have observed in W12 would contribute to remodeling of the ECM in vivo. Whereas some changes, such as up-regulation of laminin β1 (set D) and collagen XVIIα1 (set D) and down-regulation of type IV collagenase (set B), would promote deposition of ECM, other changes, such as down-regulation of TIMP1 (set B) and elastase inhibitor (set D), would facilitate ECM degradation and might contribute to stromal invasion. Integration is also associated with reduced expression of elafin (set A), an anti-leukoprotease with specific anti-elastase activity (34) that is detectable in many types of epithelium. Expression of elafin mRNA and protein in SCC has been shown to correlate inversely with grade, with strong expression in the keratinocytes of well-differentiated SCC but absence from poorly differentiated SCC in the skin (35) and esophagus (36).

Both IGF-II and EGFR showed high levels of up-regulation after HPV16 integration in W12 (both set B), with fold changes of 12.3 and 4.7, respectively. This is consistent with evidence that autocrine secretion of IGF-II, acting through the IGF-I receptor, participates in EGF-induced mitogenesis in cervical keratinocytes (37). We also demonstrated up-regulation of an EGF response factor, ERF, a zinc finger protein with unknown function (set B), which would be consistent with increased activity in the EGFR signaling pathway after episomal integration. IGF-II and EGFR may, therefore, cooperate in stimulating growth of W12 after integration and may also play a role in cervical neoplastic progression in vivo. Indeed, in one study, cervical HG-SIL and SCC were shown to express higher levels of EGFR and to be associated with elevated serum levels of IGF-II (38).

The experiments reported here were performed on W12 cells in monolayer culture, to provide a model of HPV16 infection of basal cervical squamous cells. It is likely that additional changes in gene expression would become apparent with greater epithelial differentiation. As much of the HPV life cycle is dependent on the maturation state of host keratinocytes, changes in gene expression would be likely to depend not only on the physical state of HPV but also on the differentiation state of the infected keratinocytes. Indeed, a recent study of the effects of HPV16 E6 and E7 on cervical keratinocytes (10) detected more gene expression changes in differentiating than in proliferating cells.

In conclusion, the W12 cell line has enabled us to study the effect of episomal integration on host gene expression in cervical keratinocytes naturally infected with HPV16, as a component of progression toward malignancy. We have identified functional groups of genes showing changes in expression as a result of integration and have further identified the stages in the integration process at which expression levels change. This data set can be used to direct further studies into mechanisms of progression of premalignant lesions in the cervix, and potentially at other sites.

ACKNOWLEDGMENTS

We are grateful to Richard Stephens (MRC United Kingdom HGMP Resource Centre, Hinxton, Cambridge, United Kingdom) for assistance with data analysis. We also thank Gareth Denyer (University of Sydney, New South Wales, Australia) for the use of his ChipMinerTool software and Cantab Pharmaceuticals (Cambridge, United Kingdom) for providing the anti-HPV16E7 antibody.

REFERENCES

Changes in Cervical Keratinocyte Gene Expression Associated with Integration of Human Papillomavirus 16

William Alazawi, Mark Pett, Barbara Arch, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/23/6959

Cited articles
This article cites 36 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/23/6959.full#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/23/6959.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.