Genomic imbalances in 70 snap-frozen cervical squamous intraepithelial lesions: associations with lesion grade, state of the HPV16 E2 gene and clinical outcome

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Host genomic abnormalities may determine the natural history of cervical squamous intraepithelial lesions (SILs). We undertook comparative genomic hybridisation analysis of epithelium carefully microdissected from 70 cervical SILs, the largest series to date. In contrast to previous studies, we used frozen sections for optimal DNA quality and examined whether patterns of DNA copy number imbalance (CNI) are characteristic of SIL grade, human papillomavirus (HPV) status and postoperative recurrence. We identified more CNIs in cervical SIL than previously described, with more CNIs per case in high-grade squamous intraepithelial lesion (HG-SIL) than in low-grade squamous intraepithelial lesion (LG-SIL) (P = 0.04). While some CNIs were seen at similar frequencies in HG-SIL and LG-SIL, others, including gain on 1q, 3q and 16q, were found frequently in HG-SIL but not in LG-SIL. There were significantly more CNIs per case in HG-SILs showing loss of the HPV16 E2 gene (a repressor of viral oncogene transcription) (P = 0.026) and in HG-SILs that subsequently recurred (P = 0.04). Our data are consistent with sequential acquisition of CNIs in cervical SIL progression. Higher frequency of CNI in association with E2 gene loss supports in vitro evidence that high-risk HPV integration is associated with genomic instability. Further investigation of the clinical value of specific host genomic abnormalities in cervical SIL is warranted.

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Cervical squamous cell carcinoma (SCC) usually arises from a subset of high-grade squamous intraepithelial lesions (HG-SIL), which in turn are thought to arise from a subset of low-grade squamous intraepithelial lesions (LG-SIL) (Holowaty et al., 1999; Franco et al., 2001). Untreated SILs have the potential to regress, persist or progress, and up to 8.6% of SILs recur locally following complete excision (Mohamed-Noor et al., 1997; Hulman et al., 1998; Nagai et al., 2000; Chao et al., 2004). However, the molecular pathology of cervical SILs is poorly understood and it is currently not possible to predict the natural history of an individual lesion. Unnecessary follow-up of women with cervical SILs destined not to progress or recur imposes large burdens on colposcopy services worldwide and produces substantial adverse psychosocial consequences for individual patients. There is therefore a very important requirement for new rational and objective approaches to improving diagnosis and prediction of outcome in cervical SIL.

Infection with high-risk human papillomavirus (HR-HPV) is known to be an independent risk factor for progression of LG-SIL to HG-SIL and for the development of SCC (Remmink et al., 1995; Konno et al., 1998). However, HPV testing is inherently nonspecific at identifying patients destined to develop cervical SCC (Sasieni, 2000) or HG-SIL (Lorincz et al., 2002). Other biological factors of importance in the progression of SIL or recurrence after treatment include persistence of HR-HPV (Costa et al., 2003), integration of HR-HPV into host chromosomes and the acquisition of secondary host genomic abnormalities (Lazo, 1999). Integration frequently causes disruption of the HR-HPV E2 transcriptional repressor with consequent deregulation of HR-HPV oncogenes, events that we have recently demonstrated to be important in inducing high-level genomic instability in cervical keratinocytes in vitro (Pett et al., 2004).

Very little information is available as to whether particular host genomic abnormalities in cervical SIL may be characteristic of lesion grade, HPV status or clinical outcome. Allelic imbalances have been reported on numerous chromosome arms in cervical carcinomas and small numbers of SILs (Steenbergen et al., 1996; Chu et al., 1999; Lazo, 1999; Luft et al., 2000), and there is some evidence of progressive accumulation of allelic imbalances with increasing grade of abnormality. (Larson et al., 1997; Chu et al., 1999; Luft et al., 1999; Chung et al., 2000; Lin et al., 2000; Chuaqui et al., 2001).

 Likewise, data from comparative genomic hybridisation (CGH) have been reported for only relatively small numbers of cervical
SILs (Heselmeyer et al., 1996; Kirchhoff et al., 1999, 2001; Umayahara et al., 2002). These studies have generally suggested that relatively few copy number imbalances (CNIs) exist in SIL. However, such data should be interpreted with caution, since all studies used formalin-fixed paraffin-embedded tissue, in which DNA degradation may cause difficulties with CGH, and cells microdissected under histological control do not appear to have been analysed in all studies (Kirchhoff et al., 1999, 2001). Moreover, one study combined cases equivalent to LG-SIL with cases equivalent to HG-SIL (Kirchhoff et al., 2001) and another examined SIL adjacent to SCC (Umayahara et al., 2002), which may not represent typical SIL. Most importantly, no study to date has correlated the number and type of CNIs in cervical SIL with key clinicopathological information such as HPV status and post-surgical outcome.

We have therefore performed CGH using metaphase chromosome targets to investigate the number and frequency of CNIs in a large series of 70 snap-frozen cervical SILs from 70 different patients, in which the abnormal epithelium was microdissected from serial frozen sections under histological control. None of the SILs was accompanied by SCC. We had three objectives when performing this work. Firstly, we aimed to characterise the number and locations of CNIs in cervical HG-SIL and LG-SIL. We analysed the stage of disease progression at which individual abnormalities occurred, in order to identify possible pathways of progression. We reasoned that any abnormalities present at similar frequencies in LG-SIL and HG-SIL might represent early events in the pathogenesis of SIL, while abnormalities present at a higher frequency in HG-SIL than in LG-SIL might reflect those involved in disease progression.

Secondly, we sought to identify any associations between CNI and the state of the HPV16 E2 gene in HPV16-positive HG-SILs. Initially, we performed HPV detection and typing on all cases using nested PCR and reverse line hybridisation. In HG-SILs containing HPV16, the HPV type most frequently associated with cervical SCC (Bosch et al., 1995), we examined associations between CNIs and the presence or absence of the HPV16 E2 and E7 genes, as detected by PCR. We aimed to identify cases in which HPV16 E2 was lost but HPV16 E7 was retained, as these cases would be expected to show derepression of HR-HPV oncogenes. Previous validation studies using Southern blotting had shown that such E2-negative/E7-positive cases contained HPV16 integrants in the absence of HPV16 episomes (Das et al., 1992). The E2/E7 PCR technique was employed, as the microdissected samples did not yield sufficient quantities of genomic DNA (gDNA) and RNA to enable us to use optimal techniques for assessment of HR-HPV integration, such as restriction site PCR (Thorland et al., 2000) or amplification of papillomavirus oncoprotein transcripts (Wentzensen et al., 2002). The third and final aim of our study was to assess the clinical value of selected host and viral parameters in predicting recurrence of HG-SIL after complete excision by large loop excision of the transformation zone (LLETZ).

MATERIALS AND METHODS

Tissue

The experimental work was performed with the permission of the Cambridge Local Research Ethics Committee (Ref: 03/023). The study used gDNA extracted from SIL epithelium carefully microdissected from frozen sections of cervical tissue. The tissue was obtained from LLETZ samples removed by a consultant gynaecologist from 70 different patients undergoing treatment for cervical disease. None of the cases was associated with SCC. The LLETZ samples were placed on ice after removal and study tissue was removed and snap-frozen in liquid nitrogen within 30–60 min. Samples were numbered at the time of recruitment. The histopathological diagnosis in the frozen sections for microdissection was agreed by two consultant histopathologists. Of the 70 cases, 51 were HG-SIL (given the prefix H; Figure 1) and 19 LG-SIL (given the prefix L). The median patient age was 30 years (range 19–63 years). Normal cervical epithelium and stroma were obtained from six hysterectomies performed for non-neoplastic disease unrelated to the cervix.

Identification of disease recurrence

Cases of recurrence following complete local excision were identified from review of patient records by appropriate medical practitioners. The median follow-up period was 45 months (range 11–65 months), which is appropriate for assessment of local recurrence (Mohamed-Noor et al., 1997; Hulman et al., 1998; Nagai et al., 2000; Chao et al., 2004). Recurrence was defined as a diagnosis of SIL of either grade by histology or of dyskaryosis of any grade by cytology provided the original lesion was reported as completely excised and there had been at least one negative smear and/or biopsy between the original LLETZ and the recurrence. ‘Borderline’ cytological abnormalities were not regarded as recurrences. All samples were anonymised and researchers were blinded to the available clinical data until the CGH analysis was complete.

Microdissection and DNA extraction

In all, 10 consecutive 10 μm sections were cut from each frozen tissue block. The first and last were stained with haematoxylin and eosin (H&E) and used as guides for identifying SILs in the intervening unstained sections. The abnormal epithelium was microdissected and collected using sterile scalpels under stereo-microscopic visualisation in a microdissecting microscope at ×15–25 magnification. Some sections were stained by H&E following microdissection, in order to assess the accuracy of the procedure. In all cases, at least 80% of the microdissected tissue was composed of abnormal epithelium. The microdissected tissue was placed in 50 μl of 10 mM Tris/1 mM EDTA (pH 8.0) buffer containing 0.4 μg/μl Proteinase K (Sigma, Poole UK) and incubated at 37°C overnight. Genomic DNA for HPV typing was extracted from lysates of microdissected cells using guanidinium isothiocyanate/silica as described (Boom et al., 1990).

HPV typing

PCR for HPV detection was performed as described previously (Strauss et al., 1999) using the L1 consensus degenerate primers MY09 and MY11 for the initial amplification (expected product size 452 bp) followed by a second-round nested PCR using the GP5- and GP6-positive primers (expected product size 150 bp). HPV typing was performed by reverse line hybridisation, as described elsewhere (Jordens et al., 2000), using probes complementary to sequences of the L1 region of HPV types 2, 6, 11, 16, 18, 31, 33, 35, 39, 41, 42, 43, 45, 50, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 70, 72, 81 and Han 831. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 were regarded as HR-HPV (Munoz et al., 2003), while the other types were regarded as low-risk HPV.

State of HPV16 E2 gene

Lysates of microdissected cells from HPV16-positive cases (where available) were used to assess the presence or absence of the HPV16 E2 gene, with reference to the HPV16 E7 gene. A measure of 5 μl of lysate was PCR amplified using the AmpliTaq Gold kit (Applied Bioscience, Foster City, CA, USA): 25 mM MgCl2, 2 mM dNTPs, 5 μl μl-1 AmpliTaq Gold and 20 μM primer pairs for either full-length HPV16 E2 (forward: 5'-TGGAGTGCCATCAGACACTTTGTGCGACG-3'; reverse: 5'-GCTTGTTAGAGTGTGAGCTTTGTGCGACG-3'; reverse: 2603 – 2070 British Journal of Cancer (2004) 91(12), 2063 – 2070 © 2004 Cancer Research UK
5'-TGCGATGGATTCTCATATAGACATAAATCCAG-3'; expected product size 1139 bp) or full-length HPV16 E7 (forward 5'-ATGCAATGGGATACACCTAC-3'; reverse 5'-TGGTTTCTGAGAACAGATGGG-3'; expected product size 294 bp). HPV16 DNA in the pSPHPV-16 plasmid (Stanley et al., 1989) was used as a positive control.

Samples were considered positive for a particular reaction when a band of the appropriate size was clearly identified. Cases showing...
equivocal positivity were not encountered in this study. All amplifications were preformed in triplicate. On every occasion, the presence or absence of a PCR product band was consistent for all three replicates.

Comparative genomic hybridisation

A measure of 5 μl of lysate of microdissected cells was used in a primary degenerate oligonucleotide primed (DOP) PCR reaction as described previously (Roberts et al, 1999). Test probes were made by labelling 250 ng of primary DOP products in a secondary DOP reaction incorporating digoxigenin-11-dUTP (Boehringer Mannheim, Germany). Reference DNA was obtained from normal male peripheral blood lymphocytes and subjected to two rounds of DOP–PCR, using biotin-16-dUTP in the secondary labelling reaction (Boehringer Mannheim, Germany). The test and reference DNAs were sex mismatched in order to provide an internal control.

Probes were made by ethanol coprecipitating 500 ng of test and reference products together with 5 μg of Cot-1 DNA (Roche Diagnostics, Lewes, UK). Hybridisation to normal male metaphase spreads (Vysis, Richmond, UK) was as described previously (Sanoudou et al, 2000; Roberts et al, 2001). The biotin and digoxigenin labels were detected using avidin-Cy3 (Amersham Pharmacia Biotech, Little Chalfont, UK) and antidigoxigenin fluorescein isothiocyanate (FITC)-conjugated Fab fragments (Roche Diagnostics, Lewes, UK), respectively (Roberts et al, 2001).

For each case, seven to 12 metaphases were captured on an Axioplan II epifluorescence microscope (Zeiss, Welwyn Garden City, UK) equipped with narrow bandpass filter blocks for DAPI, Cy3 and FITC, a Sensys charge-coupled device camera (Photometrics, Tucson, AZ, USA) and SmartCapture VP imaging software (Digital Scientific, Cambridge, UK). Images were assessed using Quips CGH Analysis and Interpretation software (Vysis, Richmond, UK). Nine normal–normal control hybridisations were performed using six test samples from microdissected normal ectocervical epithelium and three test samples from microdissected normal cervical stroma. Based on the results of these hybridisations, the green-to-red (test-to-reference) fluorescence intensity ratio thresholds were set to 0.85 for loss and 1.15 for gain.

Chromosomes 16p, 19 and 22 were excluded from the CGH analysis as they yield unreliable CGH data (Kallioniemi et al, 1994; Zitzelsberger et al, 1997). The telomere of chromosome 1p was not excluded, as the SIL samples showed DNA gain at this region rather than DNA loss which can represent a spurious finding; Zitzelsberger et al, 1997, with no evidence of such gain in the normal–normal hybridisations.

Statistical analysis

Differences in the number of CNIs per case between sample groups were compared using the Mann–Whitney U-test. Differences in the frequency of individual CNIs between sample groups were compared using the χ² test.

RESULTS

Clinical follow-up data

Of the 70 SILs examined by CGH, five had been inadequately excised and follow-up data were not available for four (Figure 1). In total, 61 cases (46 HG-SIL; 15 LG-SIL) were therefore completely excised and accompanied by adequate follow-up data. The median follow-up time was 45 months (range 11–65 months). Seven cases recurred (11%), of which six were HG-SIL (H16, H35, H36, H41, H50 and H51) and one was LG-SIL (L11). Recurrence was diagnosed by histological examination in all of these cases, except H36 where it was diagnosed by cytology. The median time for recurrence of the HG-SILs was 15 months (range 11–22 months).

HPV typing

Sufficient DNA was available for HPV testing following CGH in 53 of the 70 cases. HPV DNA was detected in all of these cases. In all, 15 different HPV types were detected and multiple infections were seen in 20 (38%) cases. HR-HPV types were detected in 39 of 42 (93%) of the testable HG-SILs and in eight of 11 (73%) of the testable LG-SILs (Figure 1). HPV16 was detected in 39 cases (33 HG-SIL, six LG-SIL), but only one case (an HG-SIL) was HPV18 positive.

E2/E7 PCR

A total of 23 HPV16-positive HG-SILs had sufficient residual DNA to permit PCR assessment of the presence of the HPV16 E2 and E7 genes (Figure 2). In total, 14 cases (61%) harboured HPV16 E2 but not HPV16 E2, consistent with integrated HPV16 in the absence of HPV16 episomes. Nine cases (39%) harboured intact HPV16 E2 and HPV16 E7 genes.

Comparative genomic hybridisation

Associations with lesion grade

The summary of CGH copy number karyograms for all 70 cases examined are shown in Figure 3 and the number and locations of chromosome arms involved for each case is shown in Figure 1. There were more CNI per case in HG-SIL (median 6, range 0–20; n = 51) than in LG-SIL (median 4, range 0–13; n = 19) (P = 0.04) (Figures 1 and 4a). The most frequently occurring abnormalities in the LG-SILs were gains on 1p (79%), 9q (47%), 17p (37%), 17q (32%) and 20q (32%) and loss on 4q (47%), 5q (32%), 6q (26%), 9p (26%) and 13q (21%). The most frequently occurring abnormalities in the HG-SILs were gain on 1p (80%), 17q (47%), 20q (47%), 9q (45%) and 17p (27%) and loss on 4q (53%), 6q (43%), 2q (33%), 13q (25%) and 5q (24%).

Certain consistent regions of common gain and loss were identified, particularly gain at 1pter-1p32, 3q14–21, 6p21.3–21.2 and 9q34 and loss at 2q22–32, 4q22–28, 5q14–23, 6cen-q21, 9q21, 11q12–13, 11q14–12, 12q13–12, 13q12–12 and 13q13–22. We also noted rarer gains at 3p21, 14q24 and 15q22 and rarer losses at 7p21, 7q21 and 14q12–13. No regions of amplification (test-to-reference fluorescence ratio > 1.5) were seen in any case.

Figure 5 shows the frequency of gain and loss of selected chromosome arms in all cases examined. Some CNIs occurred at essentially similar frequencies in HG-SIL and LG-SIL, including gains on 1p and 9q and losses on 4q, 5q, 6q and 13q. In contrast, gains on 1q, 3q and 16q were found frequently in HG-SIL but not...
Gain on 3q was seen in 10 cases of HG-SIL (20%) and was the only abnormality in one case. Gain of 6p was also seen frequently in HG-SIL (22%) but only in a single LG-SIL.

Associations with state of the HPV16 E2 gene in HPV16-positive HG-SIL

There were more CNIs per case in the 14 HPV16-positive HG-SIL cases with loss of the HPV16 E2 gene and retention of the HPV16 E7 gene (median 10.5, range 3–20), compared to the nine HPV16-positive HG-SIL cases with intact HPV16 E2 and HPV16 E7 (median 5, range 0–15) ($P = 0.026$) (Figure 4b). The cases with loss of E2 also showed more frequent gain on 3q ($P < 0.05$). On the other hand, we did observe high numbers of CNIs in some cases of HG-SIL in which the E2 gene was retained (e.g. case H58, which had 15 aberrant chromosome arms).

At all in LG-SIL ($P < 0.05$ for each). Gain on 3q was seen in 10 cases of HG-SIL (20%) and was the only abnormality in one case. Gain of 6p was also seen frequently in HG-SIL (22%) but only in a single LG-SIL.

Associations with clinical outcome in HG-SIL

Of the 46 cases of completely excised HG-SIL with adequate follow-up data, there were more CNIs in the six cases that were destined to recur (median 10, range 6–15) than in the 40 that did not recur (median 5.5, range 0–18) ($P = 0.04$) (Figure 4c). Loss of 4q ($P = 0.01$) and loss of 5q ($P < 0.05$) were more frequent in the HG-SILs destined to recur than in those that did not recur.

DISCUSSION

We have identified more CNIs per case than previously reported in SIL. We observed a median of four CNIs per case in LG-SIL and six CNIs per case in HG-SIL, compared, for example, to a mean of 1.1 CNIs per case in lesions equivalent to LG-SIL and a mean of 4.1...
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Number of CNIs per case according to selected clinicopathological features. Plots show the median (line), interquartile range (box) and full range (whiskers) of numbers of chromosome arms showing CNI per case in different groups of cases. There are significantly more CNIs per case in: (A) HG-SILs vs LG-SILs (P = 0.04); (B) HPV16-positive HG-SIL with disrupted HPV16 E2 vs HPV16-positive HG-SIL with intact HPV16 E2 (P = 0.026) and (C) HG-SIL destined to recur post-LLETZ vs HG-SIL that did not recur (P = 0.04).

Figure 4

Frequency of selected CNIs in LG- and HG-SILs. Bars above the x-axis indicate the percentage frequency of gains on a chromosomal arm, and bars below the x-axis indicate the percentage frequency of losses on a chromosomal arm. Yellow bars = LG-SILs; blue bars = HG-SILs.

Figure 5

CNIs per case in lesions equivalent to HG-SIL in a previous report (Umayahara et al, 2002). Our study has the advantages of using frozen sections of cervical SILs (unaccompanied by SCC) from which the lesional epithelium was microdissected, which may allow for greater sensitivity in detecting CNIs. On the other hand, it should be noted that the thresholds used for determination of copy number gain and loss have been greater in previous studies of cervical SIL (Heselmeyer et al, 1996; Kirchhoff et al, 1999; Umayahara et al, 2002).

Our data indicate that certain recurrent CNIs occur in both cervical LG-SIL and HG-SIL and suggest that there may also be sequential acquisition of imbalances during the progression from LG-SIL to HG-SIL. While the CNIs that occur at similar frequencies in LG-SIL and HG-SIL are consistent with early events, those that are significantly more frequent in HG-SIL may confer a selective advantage to LG-SIL cells and contribute to progression via clonal selection. Alternatively, the latter CNIs may represent an advantageous consequence of increased chromosomal instability in HG-SIL (Pett et al, 2004) and be of greater relevance in favouring subsequent progression to SCC.

CGH losses reported here are consistent with sites of allelic imbalance previously reported in cervical SIL and SCC (Lazo, 1999), including 3p12 – 3p14.1 (Larson et al, 1997; Chu et al, 1999; Chung et al, 2000), 4p16 (Larson et al, 1997), 4q21 – q35 (Larson et al, 1997), 6p14 – 22 (Chatterjee et al, 2001; Steenbergen et al, 2001), 6q22 – q27 (Chuaqui et al, 2001), 9p21 (Lin et al, 2000), 11p14 – 15 and 11q23 (Lutfi et al, 1999; Pulido et al, 2000). Further investigation of these sites of potential tumour suppressor genes is warranted.

We observed gain of 3q in 20% of 51 HG-SIL cases. Whereas this imbalance was initially described as defining the transition to SCC in the cervix (Heselmeyer et al, 1996), it was subsequently reported in eight of 37 (22%) paraffin-embedded ‘dysplastic’ lesions equivalent to HG-SIL (Kirchhoff et al, 1999; Kirchhoff et al, 2001), an observation that is supported by our data. Interestingly, we found that six of the 10 HG-SILs in our study that showed gain of 3q also showed loss on 3p or 13q, also supporting the previous suggestion of an association between these abnormalities (Kirchhoff et al, 1999). Our findings are consistent with the earlier identification of 3q25 – q27 as the consensus region of gain.

Cases of HPV16-positive HG-SIL in which the E2 gene was disrupted showed significantly more CNIs and significantly more frequent gain on 3q than cases in which the E2 gene was intact. Data from the PCR approach that we used to detect E2 and E7 have previously been shown to correlate with the physical state of HPV in clinical specimens, as determined by Southern blotting (Das et al, 1992). Loss of E2 (with retention of E7) is consistent with the presence of integrated HPV16 in the absence of HPV16 episomes. The presence of the HPV16 E2 gene could either be due to episomal HPV16 or to integrated HPV16 in which the gene is...
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