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Original Paper

Increased gene copy numbers at chromosome 20q are frequent in both squamous cell carcinomas and adenocarcinomas of the cervix

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Abstract

Genome-wide microarray-based comparative genomic hybridization (array CGH) was used to identify common chromosomal alterations involved in cervical carcinogenesis as a first step towards the discovery of novel biomarkers. The genomic profiles of nine squamous cell carcinomas (SCCs) and seven adenocarcinomas (AdCAs), as well as four human papillomavirus (HPV)-immortalized keratinocyte cell lines, were assessed. On a genome-wide scale, SCCs showed significantly more gains than AdCAs. More specifically, there was a striking and highly significant difference between the two histological types for gain at 3q12.1–28, which was predominantly observed in SCC. Other frequent alterations included gains of 1q21.1–31.1 and 20q11.21–13.33, and losses of 11q22.3–25 and 13q14.3–21.33. Subsequent FISH analysis for *hTR*, located at 3q26, confirmed the presence of 3q gain in SCCs and HPV-immortalized cell lines. Fine mapping of chromosome 20q using multiplex ligation-dependent probe amplification (MLPA) showed copy number increases for a number of genes located at 20q11–q12, including *DNMT3B* and *TOP1*. For *DNMT3B*, this correlated with elevated mRNA expression in 79% of cases. In conclusion, the assessment of frequent genomic alterations resulted in the identification of potential novel biomarkers, which may ultimately enable a better risk stratification of high-risk (hr)-HPV-positive women.

Keywords: cervical cancer; HPV; array CGH; MLPA; 20q; 3q; DNMT3B

Introduction

Cervical carcinoma is the second most common malignancy in women worldwide. Approximately 80% of these tumours are squamous cell carcinomas (SCCs) and 5–20% are adenocarcinomas (AdCAs) [1,2]. Recent studies indicate that although the presence of cervical screening programmes has resulted in a decrease in the incidence of SCC in developed countries, the incidence of AdCA has remained the same or even increased [3–6].

Infection with high-risk (ie oncogenic) human papillomaviruses (hr-HPVs) is causally involved in cervical carcinogenesis. Several studies have demonstrated that adding hr-HPV DNA testing to cervical cytology results in a negative predictive value approaching 100% for cervical intraepithelial neoplasia grade 3 lesions and cervical carcinoma (≥CIN 3) [7–12]. The positive predictive value of hr-HPV testing, however, is limited. Next to persistent hr-HPV infection, specific (epi)-genetic changes in the host-cell genome are essential for the development of ≥CIN 3 [13–15]. Gaining more insight into common chromosomal alterations present in cervical carcinomas could therefore lead to the discovery of novel biomarkers that can distinguish hr-HPV-positive women with ≥CIN 3. Previous genome-wide studies, using classical comparative genomic hybridization (CGH), showed recurrent losses on chromosomes 2q, 3p, 4p, 5q, 6q, 11q, 13q, and 18q, and gains of 1q, 3q, 5p, 8q, and Xq [16–23]. A number of these alterations are already evident in HPV-immortalized keratinocytes, which are reminiscent of pre-malignant cervical lesions when cultured on collagen rafts [24–26].

Most studies have focused predominantly on cervical SCC and it is unclear to what extent the results obtained are also representative of AdCA. To identify common genetic alterations in cervical cancer, we determined high-resolution chromosomal signatures of both cervical SCC and AdCA, using microarray-based CGH (array CGH) with an average resolution of 1 Mb [27]. The BACs used in array CGH are representatives of a chromosomal region and as such, are independent of gene density or regulation [28]. The high resolution

in combination with frozen specimens allowed us to determine more detailed genomic profiles than was previously possible. In addition, HPV-immortalized cell lines were analysed to identify genomic alterations that may already be present in non-invasive precursor lesions. Finally, one chromosomal region (ie 20q) that appeared frequently amplified was chosen for further fine mapping using multiplex ligation-dependent probe amplification (MLPA) to gain more insight into the genes that may be affected by this alteration [29].

Materials and methods

This study followed the ethical guidelines of the Institutional Review Board of the VU University Medical Center.

Cell lines

Establishment and culture of the HPV16 (FK16A/FK16B) and HPV18 (FK18A/FK18B) immortalized cell lines have been described previously [30]. Primary human keratinocytes, referred to as EK cells, were isolated from foreskin and cultured as described previously [30]. The human cervical carcinoma cell lines SiHa, HeLa, and CaSki were obtained from the American Type Culture Collection (Manassas, VA, USA).

Tissue specimens

We used frozen specimens from 16 SCCs, seven AdCAs, and three histologically normal cervices distant from tumour, all of which were collected during the course of routine clinical practice at the Department of Obstetrics and Gynaecology at the VU University Medical Center (Amsterdam). The average patient age in the tumour group was 46 years (range 25–75 years) and did not differ significantly between patients with SCC and AdCA (Table 1). Furthermore, DNA was extracted from whole blood samples from healthy men to be used as normal reference DNA in both array CGH and MLPA experiments, and lymphocytes were isolated for FISH analysis. Sequential series of cryo-sections were used for DNA/RNA extraction from frozen normal cervical specimens and tumour specimens containing more than 70% tumour cells. Tumour specimens containing less than 70% tumour cells were first enriched by means of laser capture microdissection using a Leica ASLMD microscope (Leica, Heidelberg, Germany).

Extraction of nucleic acids and HPV typing

Total RNA was isolated using TRIzol Reagent (Life Technologies, Breda, The Netherlands) according to the manufacturer's instructions. Following RNA extraction, DNA was extracted from samples containing more than 70% tumour and normal specimens following the manufacturer's recommendations (http://www.vumc.nl/microarrays/). After microdissection, genomic DNA was extracted by proteinase K digestion followed by standard phenol–chloroform extraction as described previously [31]. Genomic DNA from cell lines and whole blood samples was extracted using the Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands) and Qiagen maxi blood kit (Qiagen, Leusden, The Netherlands), respectively. Human placental DNA (HP-DNA) was purchased from Sigma-Aldrich (St Louis, MO, USA).

HPV typing was performed using the general primer GP5+/6+ polymerase chain reaction (PCR) followed by reverse line blot, as described by Van den Brule *et al* [32].

Table 1. Summary of clinical data and HPV typing of the carcinomas analysed

Tumour sample	Differentiation grade	Tumour stage	HPV type	Age (years)
SCC 4	MD	IIA	67	62
SCC 7	PD	IIIB	16	37
SCC 11	PD	IIB	16	75
SCC 12	PD	IB	16	44
SCC 15	PD	IB	16	47
SCC 21	MD	IB	16	44
SCC 27	MD	IB2	69	49
SCC 28	MD	IB2	35	48
SCC 32	MD	IIA	16	37
SCC 36	MD	IIA	16	72
SCC 38	MD	IB1/IIA	16	51
SCC 39	PD	IB1	33	40
SCC 47	PD	Unknown	16	45
SCC 48	PD	Unknown	16	53
SCC 51	PD	Unknown	16	25
SCC 55	PD	IB	HR+	38
AdCA 1	WD	IB	16	34
AdCA 2	WD	IB	16	35
AdCA 5	MD	IB	16	31
AdCA 9	MD	IB	16	45
AdCA 10	MD	IB2	16	39
AdCA 11	MD	IB	18	64
AdCA 12	MD	IB2	18	41

WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated; HR+ = high risk-positive.

CGH microarrays

Tumours were hybridized on CGH BAC microarrays, containing 4202 BAC clones spotted in triplicate, produced at the VUmc Microarray facility (http://www.vumc.nl/microarrays/) [33]. Cell lines were hybridized on arrays manufactured and hybridized at the UCSF Cancer Research Institute, which contained 2464 BAC clones (HumArray 1.14 [34]). Tumour hybridizations and cell line hybridizations were performed as described by Snijders *et al* [34]. For tumour samples, both pre-hybridization and hybridization were performed in a hybridization station (HybStation12, Perkin Elmer Life Sciences) [33].

Fluorescence *in situ* hybridization (FISH)

FISH analysis of the *hTR* gene and *hTERT* was conducted using the P1 probes 9913 and 518 C13, respectively, as described previously [35]. *hTR* locus copy numbers were determined in interphase nuclei from eight SCCs, two normal cervices, and HPV-immortalized cells. In addition, *hTR* and *hTERT* copy numbers were determined in metaphase spreads from HPV-immortalized cells and normal lymphocytes. For each analysis, 100 nuclei and 50 metaphase spreads were scored, respectively.

Multiplex ligation-dependent probe amplification (MLPA)

For the present study, a dedicated oligonucleotide MLPA probe set for 36 genes developed by MRC-Holland (http://www.mrc-holland.com/) was used. The probe set included probes for 24 genes located on chromosome 20, largely based on the probe set described by Postma *et al*, as well as 12 probes for reference genes [36]. The latter genes were selected from loci showing no or only infrequent array CGH alterations and were used for normalization purposes. MLPA reactions were performed as described previously [29,36].

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed using *DNMT3B* and *snRNP U1A* specific primers. The latter served as a reference for the semi-quantitative assessment of *DNMT3B* mRNA levels [37,38].

Data analysis

Image acquisition and quantification

Array CGH slides were scanned using ScanArray Express (Perkin Elmer Life Sciences) and quantified in ImaGene 5.6.1 software (BioDiscovery Ltd, Marina del Rey, CA, USA) using default settings for the flagging of poor quality spots. Image acquisition and analysis of the HumArray 1.14 cell line hybridizations were carried out using a custom-built CCD camera imaging system [39]. Analysis of the array images with automatic feature extraction and subsequent data analysis was performed with dedicated software UCSF SPOT and SPROC [40].

Array analysis

BAC clones were positioned along the genome according to the May 2004 freeze. After exclusion of flagged spots, the average of the triplicate spots was calculated for each BAC clone and 2 log ratios (tumour/normal reference signal) were normalized by subtraction of the mode value of BAC clones on chromosomes 1–22. BAC clones with flagged spots were excluded from analysis. In order to determine exact breakpoints in the generated CGH profiles, we smoothed the obtained 2 log ratios using aCGHsmooth software [41]. A smoothed 2 log ratio between -0.15 and 0.15 was considered normal. These thresholds were determined by smoothing 15 normal-to-normal hybridizations and calculating 99% confidence intervals (99% CIs). Only gains and losses consisting of at least three consecutive BAC clones were included.

MLPA analysis

For each sample, peak areas of every probe were determined in triplicate for further analysis. Normal reference peak areas were obtained from normal lymphocytes ($n = 5$), normal frozen cervical samples ($n = 3$), primary keratinocytes ($n = 1$), and human placenta DNA (Sigma-Aldrich). Tumour (cell line) to normal DNA copy number ratios were obtained by dividing the area under the peak for each probe in the tumour by the mean value of the same peak in the normal control samples. Ratios were normalized by setting the median ratio of the reference probes to 1.0. For each probe, 99% CIs were determined in the normal control samples, and genes with a ratio (including standard deviation) outside this 99% CI were considered to be gained or lost, respectively.

Statistical analysis

The number of aberrations was compared between SCC and AdCA specimens using the non-parametric Mann–Whitney test. To determine chromosomal regions with a significantly different alteration pattern between SCC and AdCA, we applied CGH-MultiArrayRegion software (http://www.win.tue.nl/~markvdw/CGHMultiArray.html), which contains a Wilcoxon two-sample test statistic with ties and includes a false discovery rate (FDR) correction for multiple testing, needed to discriminate real differences from chance effects [42]. We used the following region determining settings: dmax2 = 5, dmax3 = 3, dfirstlast = 1000, delsingle = 1. Two-sided p values of less than 0.05 (including FDR correction) were considered statistically significant.

Results

Distinct genomic signatures in SCC and AdCA

Sixteen cervical carcinomas, nine SCCs and seven AdCAs, were analysed using array CGH to determine the chromosomal aberrations present. A summary of all the chromosomal aberrations found is shown in Table 2. On average, 10.1 aberrations (range 0–22) were found per tumour, with a mean number of 5.7 gains (range 0–14) and 4.4 losses (range 0–13). A significantly higher number of gains were found in SCCs (average 7.7 gains; range 3–14) than in AdCAs (average 3.1 gains; range 0–5; $p = 0.005$).

Table 2. Summary of the chromosomal alterations detected in cervical carcinomas by array CGH analysis

Tumour sample histology (HPV type)	Gained regions	Lost regions	Amplifications
SCC 4 (67)	1p36.33–31.3, 1q12–44, 3q12.1–29, 7p22.3–q36.3, 9p24.3–q34.3, 12q22–24.33, 16q21–24.3, 20p13–q13.33	8q23.2–23.3, 11p15.5–14.1, 11q14.3–25, 13q14.3–21.33, 13q31.1–31.3, 18q22.1–23	
SCC 12 (16)	1q12–44, 3q12.1–29, 11q23.3, 12q13.11–13.2, 20p13–q13.33, 21q11.2–22.3	1p31.2–31.1, 2q34–37.3, 8q23.2–24.11, 10p15.3–q26.3, 11p15.5–q11, 13q12.11–31.1, 17p13.3–11.2	
SCC 15 (16)	1q12–31.1, 3q12.1–29, 20q11.21–13.33, 21q11.2–22.3, 22q11.1–13.33	1p31.2–31.1, 10p15.3–q26.3, 13q12.11–31.1	
SCC 27 (69)	1p36.33–36.21, 3q13.11–29, 11q12.1–22.3, 14q31.3–32.33, 20p13–q13.33	3p14.2–q12.3, 5q11.1–35.3, 8q23.1–24.13, 11q22.3–25, 13q12.11–34, 18p11.22–11.21	
SCC 28 (35)	1q12–44, 3p26.3–21.1, 3q12.1–29, 11p13–12, 11q23.3, 14q11.2–32.33, 20q11.21–13.33	11p15.2–p13	
SCC 32 (16)	1q12–44, 3q13.2–29, 5p15.32–12, 9p23–22.3, 11q14.1–22.3, 12p12.1–11.23, 17q21.31–25.3, 19q12–13.43, 20p13–q13.33	1p36.33–36.32, 2p11.2–37.3, 3p26.3–q13.13, 4p16.3–q35.2, 7p22.3–36.3, 10p15.3–q26.3, 11q22.3–25, 14q11.2–32.33, 16p13.3–11.2, 17p13.3–11.2, 19p13.3–12, 21q22.3, 22q11.1–13.33	11q22.2–22.3, 12p12.1–11.23
SCC 36 (16)	1p36.33–22.1, 1q12–44, 2p25.3–11.2, 3p26.3–q29, 5p15.33–35.3, 6p24.1–22.3, 6p22.2–q11.1, 7p11.2, 11q12.1–13.4, 14q24.2–32.33, 15p13–q13.1, 19q12–13.43, 20p13–q13.33, 21q11.2–22.3		6p21.2, 7p11.2
SCC 38 (16)	1p36.33–31.1, 3p11.2–q29, 16p13.2–13.12	10p15.3–q26.3, 15q11.2–15.1, 17p13.3–q11.1	
SCC 39 (33)	1p36.33–q44, 3p11.2–q29, 4p16.3–q35.2, 5p15.33–q35.3, 6q27, 11p15.5–q13.5, 11q22.1–22.3, 12p13.33–13.2, 17p13.3–q25.3, 18p11.32–q23, 20p13–q13.33, 21q11.2–22.3	11q14.1–22.1, 11q22.3–25	12p13.33–13.31
AdCA 1 (16)	1p36.33–31.3, 1p13.3–q44	6p12.3–q16.3, 8q22.3–23.3, 10q11.23–21.3, 13q14.3–21.33, 13q31.1–31.3, 21q11.2–21.3	
AdCA 2 (16)	1p36.31–31.1, 2p25.3–q13, 7p22.3–13, 12q13.11–14.1, 20p13–q13.33	7q11.23–36.1, 11q14.1–25, 12p13.33–12.1, 13q12.11–34, 21q11.2–22.11, 21q22.3	
AdCA 5 (16)	1p31.1, 1q12–44, 3q25.31–29, 12q24.33	18q12.1–23	
AdCA 9 (16)			
AdCA 10 (16)	1p36.32–q44, 19q13.31–13.32	8q23.2–23.3, 9q21.31–31.1, 11q22.1–25, 13q12.11–34, 16p12.1–q24.3	
AdCA 11 (18)	1p31.1, 1q12–44, 8q13.2–24.3, 11p15.4–14.1, 11q13.3–13.4	8q24.3, 11q11–12.3, 11q22.1–25, 13q12.3–34, 14q32.33, 16q24.2–24.3, 17q25.3, 18q23, 20q13.33	11q13.3–13.4
AdCA 12 (18)	3p26.3–q29, 13q12.11–34, 18p11.32–q11.1, 20p13–q13.33	4p16.3–q35.2, 5q35.1–35.3, 17q25.1–25.3	

To visualize both commonly and specifically altered chromosomal regions in cervical SCC and AdCA, the frequency of gains and losses per BAC was plotted (Figure 1). Frequent alterations (>25% of cases) include gains on chromosomes 1, 3, and 20, and losses on chromosomes 8, 10, 11, and 13 (Figure 1A). To determine the smallest regions of overlap (SRO), we aligned all the carcinoma samples, which resulted in five gained SRO and five lost SRO (Table 3).

Since SCCs showed a significantly higher number of alterations compared with AdCAs, we wondered whether this could be attributed to specific chromosomal regions displaying different

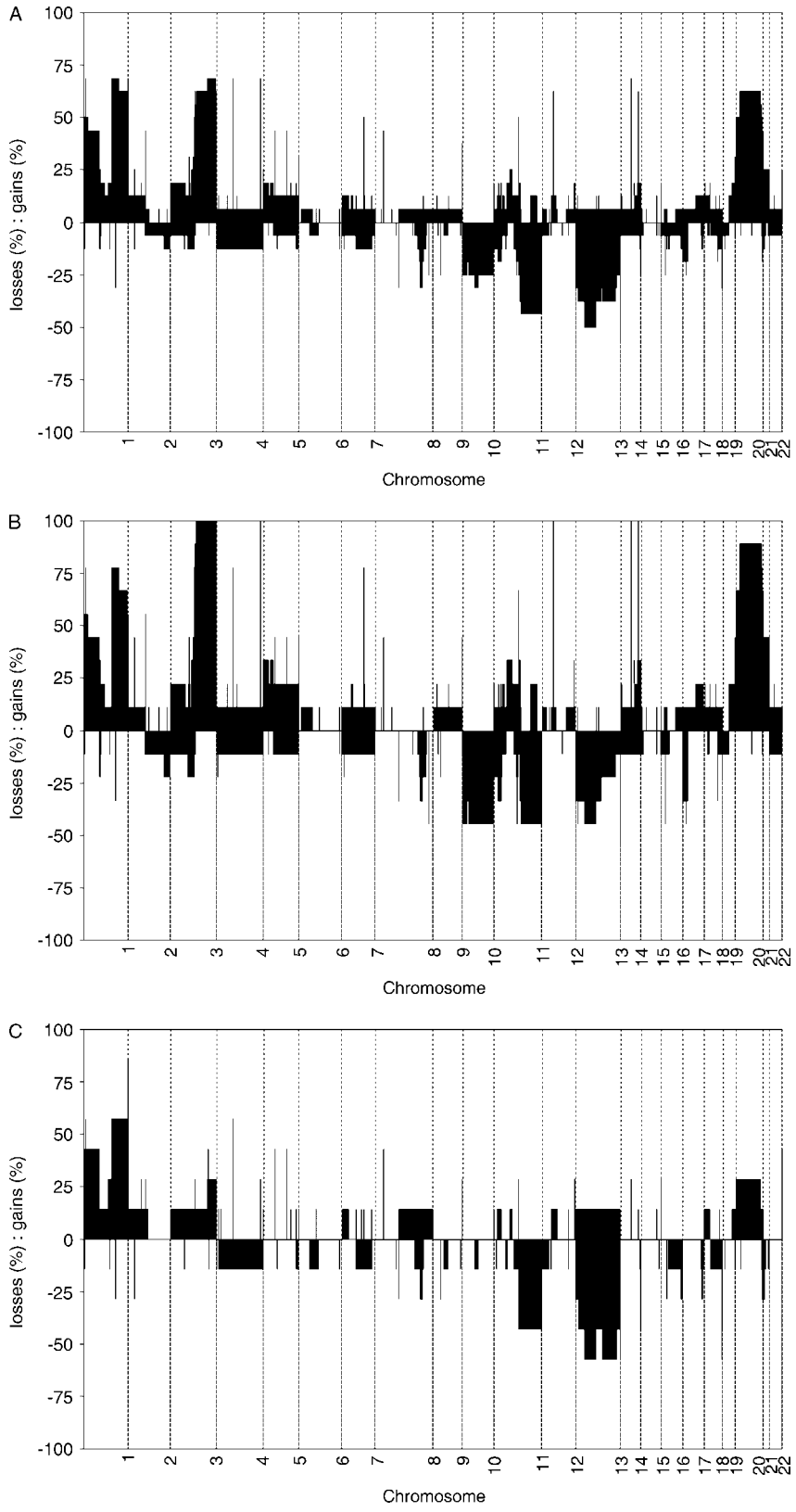

Figure 1. Frequency plots of gains and losses for chromosomes 1–22 as determined by array CGH. Percentages of gains (positive axis) and losses (negative axis) are shown for each BAC clone in (A) all carcinomas analysed, (B) SCCs, and (C) AdCAs

patterns in SCC compared with AdCA. Using CGHMultiArrayRegion software, we found that gains of chromosome 3q12.1–28 were significantly more frequent in SCC ($p = 0.002$, FDR = 0.019).

Differences between histological types are unrelated to HPV type

To determine whether differences found between SCC and AdCA were a consequence of differences in

Table 3. Percentages of frequently altered regions in cervical carcinomas. The regions mentioned here were altered in more than 25% of cases and include the smallest regions of overlap (SRO) between samples

Chromosomal region	Gain/loss	% total (*n*)	% SCC (*n*)	% AdCA (*n*)
1p36.31–36.21	Gain	50 (8)	56 (5)	43 (3)
1p31.1	Gain	38 (6)	33 (3)	43 (3)
1q12–31.1	Gain	69 (11)	78 (7)	57 (4)
3q25.31–29	Gain	69 (11)	100 (9)	29 (2)
20q11.21–13.33	Gain	63 (10)	89 (8)	29 (2)
8q23.2–23.3	Loss	31 (5)	33 (3)	29 (2)
10q11.23–21.3	Loss	31 (5)	44 (4)	14 (1)
11q22.3–25	Loss	44 (7)	44 (4)	43 (3)
13q14.3–21.33	Loss	50 (8)	44 (4)	57 (4)
13q31.1–31.3	Loss	38 (6)	22 (2)	57 (4)

HPV-type distribution, we next compared tumours based on HPV type. All carcinomas were hr-HPV-positive: HPV16 in five SCCs and 5 AdCAs; HPV18 in two AdCAs; and HPV33, 35, 67, and 69 in one SCC each, respectively. The mean number of aberrations, gains, and losses did not differ significantly between HPV16-positive and HPV16-negative tumours. In addition, using CGHMultiArrayRegion, we did not find significantly different chromosomal patterns related to HPV type.

When comparing exclusively HPV16-positive SCCs with AdCAs ($n = 10$; five SCCs and five AdCAs), the mean number of gains was again significantly higher in SCC [$p = 0.032$; SCC: 7.0 gains (range 3–12); AdCA: 2.6 gains (range 0–5)]. A gain at 3q12.1–28 also remained significantly more frequent in HPV16-positive SCC than in HPV16-positive AdCA ($p = 0.008$; FDR = 0.024). These results indicate that the differences found in chromosomal aberrations between SCC and AdCA are unrelated to the HPV type.

Frequent alterations in HPV-mediated immortalized cell lines and cervical cancer cells

To identify genomic alterations that may reflect high-grade pre-malignant lesions, four HPV-immortalized primary human foreskin keratinocyte cell lines were analysed by array CGH as well. Alterations common to all cell lines included gains of chromosomes 3q, 5p, and 20q, as well as a loss of chromosome 22q. All of these alterations were also observed in the cervical cancer cell line SiHa. Moreover, in SiHa cells, a narrow high-level amplification was observed at 20q11.22 (Table 4).

A comparison between the genomic profiles in HPV-immortalized cell lines and cervical carcinomas revealed gains of 3q25–29 (69% of carcinomas; 100% of cell lines), 20q11.21–13.33 (63% of carcinomas; 100% of cell lines), and loss of chromosome 13q14.3–21.33 (50% of carcinomas; 50% of cell lines) to be frequent in both cell lines and carcinomas.

Table 4. Summary of the chromosomal alterations detected in the HPV-immortalized cell lines (FK16A, FK16B, FK18A, and FK18B) and SiHa as determined by array CGH analysis

Cell line (HPV type)	Gained regions	Lost regions	Amplifications
FK16A (16)	3q13.11–29, 5p15.33–13.1, 20q11.22–13.33	2p25.2–q37.3, 3p26.3–12.2, 4p16.3–35.1, 6p25.1–22.1, 6p11.2–q12, 8p23.3–q12.1, 10q21.2–26.3, 11p15.5–11.2, 13p13–q33.1, 15p12–q25.1, 16p13.2–11.1, 18p11.32–q23, 19p13.3–q12, 21p12–q21.3, 22p11.2–q12.3	
FK16B (16)	3q13.11–29, 4p15.2–14, 5p15.33–q35.3, 6p25.1–q12, 7p22.3–q36.3, 8q12.1–24.3, 9p24.3–q32, 11p13–11.12, 11q13.2–25, 14p13–q32.2, 19p12–q13.43, 20p13–q13.33	4p16.3, 4p11–q35.1, 10p15.3–q11.23, 10q26.13–26.3, 11p15.5–14.3, 15q21.1–25.1, 22p12–q12.3	
FK18A (18)	3q13.11–29, 5p15.33–q35.3, 7p22.3–12.1, 8q12.1–24.3, 9p24.3–q32, 10p15.3–q26.3, 11p15.5–q25, 13p12–q33.1, 20p13–q13.33, 21p12–q21.3, 22p13–q11.1	22q11.21–12.3	
FK18B (18)	1q12–43, 3q25.1–29, 5p15.33–12, 7p22.3–q36.3, 8q12.1–24.3, 9p24.3–q32, 10p15.3–11.21, 11q11–24.3, 14q21.1–32.2, 19p12–q13.43, 20p13–q13.33, 21p12–q21.3, 22p13–q11.1	3p26.3–12.2, 4q22.1–35.1, 8p23.3–q12.1, 13p13–q33.1, 22q11.22–12.3	
SiHa (16)	3q24–25.31, 5p15.33–12, 8q24.21–24.3, 9q22.31–32, 15q21.2–25.1, 16q12.1–22.1, 16q23.2–24.1, 19p12–q13.43, 20q11.22–13.33, 21q21.1–21.3	1p22.3–22.2, 2q36.1–37.3, 4p16.3–q35.1, 6p25.1–q25.1, 7q11.21–11.22, 8p23.3–q24.13, 9p24.2–21.1, 10p11.23–q11.23, 13p12–q32.3, 16q22.1–23.1, 18q12.3–23, 22p13–q12.3	20q11.22

Table 5. Summary of *hTR* locus copy numbers as determined by FISH

Sample	Mean number of *hTR* signals/ nucleus	% of nuclei with >2 *hTR* signals/nucleus	Range of *hTR* signals/ nucleus
Normal lymphocytes	2	0	2
Normal cervix 1	1.26	0	0–2
Normal cervix 2	1.59	10	0–4
SCC 7	2.64	47	0–9
SCC 11	3.07	60	0–8
SCC 21	5.27	94	1–14
SCC 27	3.02	62	0–8
SCC 47	2.47	42	0–5
SCC 48	3.91	76	0–9
SCC 51	3.40	70	0–8
SCC 55	2.84	63	1–7
FK16A	4.80	72*	3–7
FK16B	4.70	68*	3–7
FK18A	4.20	28*	3–6
FK18B	4.28	32*	3–7

* % of nuclei with more than 4 *hTR* signals/nucleus.

Gain of 3q is associated with increased *hTR* copy numbers

To confirm the array CGH results, we performed FISH analysis using a probe for the *hTR* gene located at the frequently gained region 3q26. In contrast to normal lymphocytes and normal cervices, all SCCs ($n = 8$) revealed three or more copies of *hTR* in over 40% of nuclei analysed (Table 5; see example in Figure 2A). Moreover, in all four HPV-immortalized cell lines, all of which are tetraploid, at least 28% of nuclei were shown to contain five or more copies of *hTR* (Figure 2B and Table 5). FISH analysis of metaphase spreads from the HPV-immortalized cells using a second probe specific for *hTERT*, located at 5p15, also revealed elevated *hTERT* copy numbers in these cells (Figure 2B). This provides additional confirmation of the array CGH results, which also revealed a gain of 5p in all HPV-immortalized cell lines (Table 4).

Fine mapping of the frequently gained region on chromosome 20q

Our array CGH data revealed highly frequent gains of 3q and 20q in both cervical carcinomas and HPV-immortalized cell lines, indicating that these alterations may occur rather early during cervical carcinogenesis. As gain of 3q has already been described previously in cervical carcinomas and candidate oncogenes, ie *hTR* and *PIK3CA* have already been identified [18,43], we focused on the newly found chromosomal gain at 20q, for which no candidate oncogenes involved in cervical carcinogenesis are yet known. Therefore, MLPA, a recently developed PCR-based method to study copy number alterations of individual genes, was performed.

Normal control samples ($n = 10$) and a pre-immortal passage of the FK18B cell line showed no copy number alterations in any of the 36 genes studied (Figure 3). MLPA analysis of HPV-immortalized cell lines, SiHa cells, and 15 of the 16 carcinomas studied by array CGH revealed frequent copy number increases for a number of genes located within the 20q11.1–12 region, namely *REM1, DNMT3B*, *E2F1*, and *TOP1*. The latter showed increased copy numbers in all carcinomas and immortalized cell lines analysed. In addition, *ADA*, which is located at 20q13.12, and *THBD*, which is located on 20p, often show increased copy numbers (Figure 3). Some of these genes also revealed increased copy numbers in carcinomas that did not display a gain at chromosome 20 by array CGH, which might be explained by the higher resolution of MLPA for individual genes. Occasionally, reference genes used in the MLPA assay showed copy

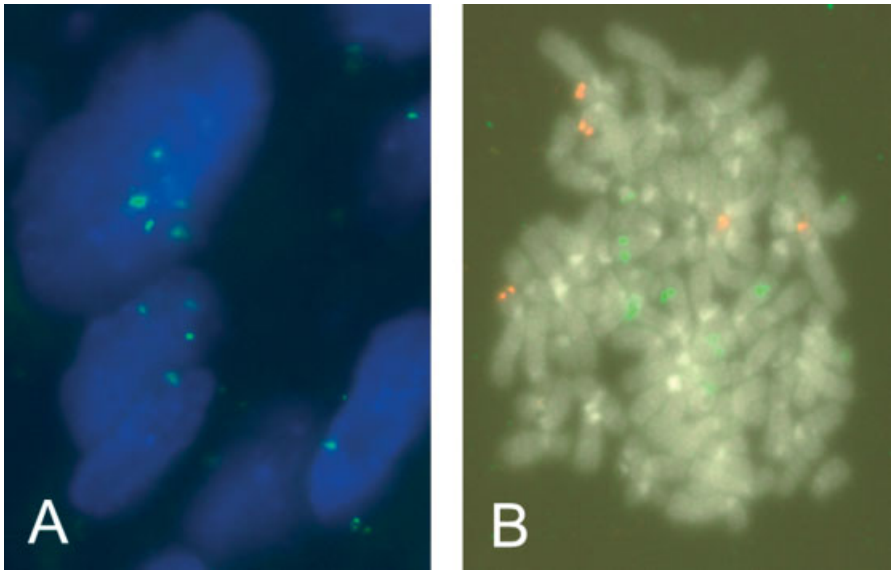

Figure 2. Fluorescence *in situ* hybridization (FISH) analysis of (A) SCC 21 with a probe for *hTR* (located at 3q26; green) and (B) a metaphase spread from cell line FK18B with a probe for *hTR* (located at 3q26; green) and a probe for *hTERT* (located at 5p15; red)

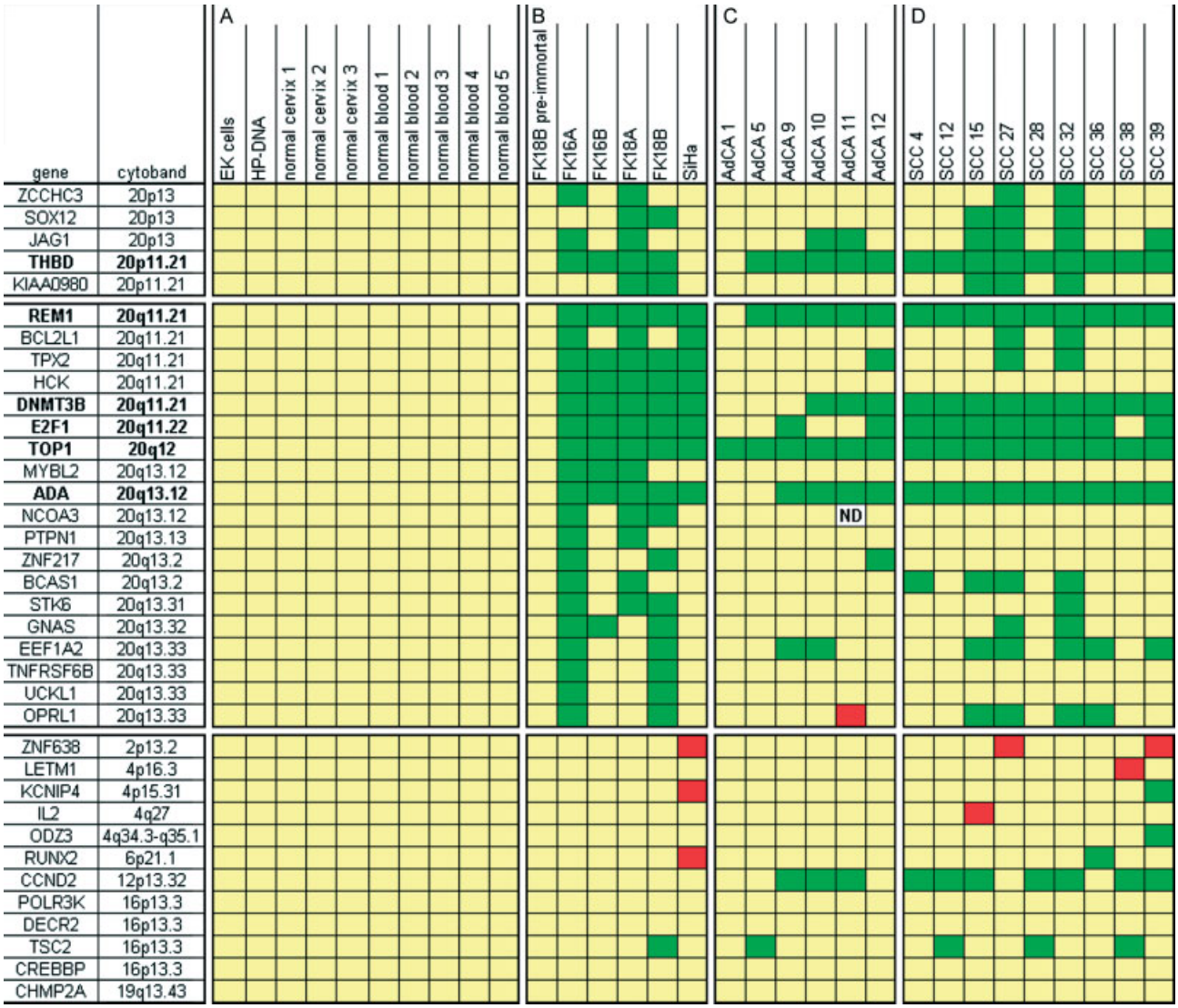

Figure 3. Copy number changes for genes located at chromosome 20p, 20q, and control genes located throughout the genome as determined by MLPA analysis in (A) normal controls primary foreskin keratinocytes (EK cells), human placental DNA (HP-DNA), normal cervices and normal blood samples; (B) a pre-immortal passage of the FK18B cell line and four HPV-immortalized cell lines (FK16A, FK16B, FK18A, and FK18B); (C) six AdCAs; (D) nine SCCs. Copy number increases are depicted in green; copy number decreases in red; and normal copy numbers in yellow. ND = not done

number alterations, some of which were accompanied by array CGH aberrations. This, however, did not interfere with the ratios ultimately calculated for the chromosome 20 genes.

Increased *DNMT3B* copy numbers correlate with elevated mRNA levels

The gene *DNMT3B* showed copy number increases in all HPV-immortalized cell lines, all SCCs, and 50% of the AdCAs analysed, and a high-level amplification in SiHa cells. To determine whether increased copy numbers of *DNMT3B* are reflected by elevated mRNA expression, semi-quantitative RT-PCR was performed on three cervical cancer cell lines (SiHa, HeLa, and CaSki), two HPV-immortalized cell lines (FK16A and FK18B), nine SCCs, and four AdCAs, from which good-quality RNA could be extracted (Figure 4). Elevated expression of *DNMT3B* was detected in SiHa, CaSki, FK16A, seven of nine SCCs, and three of four AdCAs. Elevated gene copy numbers and mRNA expression levels of *DNMT3B* were found to correlate in 11 of 14 (79%) samples. In one AdCA, elevated mRNA levels were detected even though no increased gene copy numbers were found. In FK18B and SCCs 12 and 36, on the other hand, *DNMT3B* mRNA expression was not elevated, despite the fact that the gene showed increased copy numbers in the MLPA analysis (Figure 3).

Discussion

This study was initiated to determine common chromosomal alterations in cervical cancer, with a higher resolution than has been possible so far, as a first step in the identification of novel biomarkers for a better risk stratification of hr-HPV-positive women. We

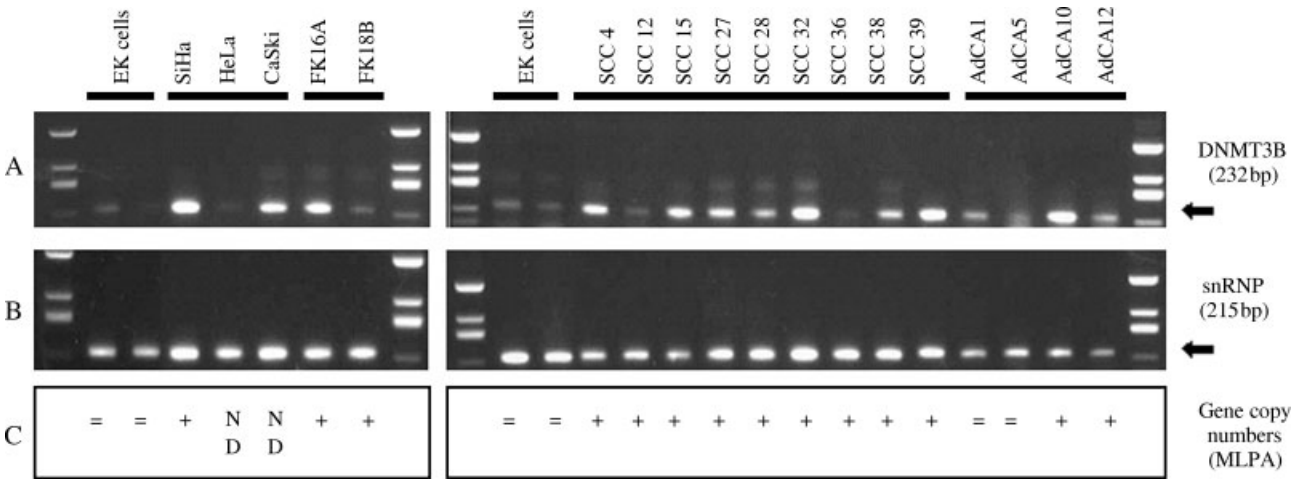

Figure 4. (A) *DNMT3B* mRNA expression levels were determined in three cervical cancer cell lines (SiHa, HeLa, and CaSki), two HPV-immortalized cell lines (FK16A and FK18B), nine SCCs, and four AdCAs by semi-quantitative RT-PCR. (B) *U1A snRNP* expression levels are included as a housekeeping control. (C) *DNMT3B* gene copy numbers as determined by MLPA are shown; +: copy number increase; =: no copy number change; ND: not done

included both SCC and AdCA of the cervix as well as HPV-immortalized cell lines, the genomic profiles of which reflect rather early events in cervical carcinogenesis [25,26].

SCCs showed significantly higher numbers of gains compared with AdCAs ($p = 0.005$), which could primarily be attributed to a gain of 3q12.1–28 (FDR-corrected $p = 0.019$). When considering HPV16-positive tumours solely, differences between SCC and AdCA remained significant, indicating that HPV type does not interfere. Since other HPV types were only present in a small subset of tumours analysed, we were unable to determine whether this difference would also be evident for tumours containing HPV types different from HPV16. From several studies it can be concluded that HPV18 is the predominant type associated with AdCA, whereas in our study only two out of seven AdCAs were HPV18-positive [44,45]. Although this might have influenced our analyses, our data suggest that genomic profiles of HPV18-positive AdCA do not necessarily differ from those of HPV16-positive AdCA. At present, it cannot be excluded that the chromosomal signatures related to other HPV types differ from those of HPV16. Analysis of a larger series of carcinomas containing different HPV types is necessary to identify possible HPV type-specific chromosomal alterations.

In cervical cancer, as well as a number of other solid tumours, a gain of chromosome 3q is described as one of the most consistently found alterations. Within this region, two candidate oncogenes are located: *hTR*, which encodes the RNA component of telomerase; and *PIK3CA*, which is involved in the PI3-kinase/AKT signalling pathway and has been proposed to act as an oncogene in cervical cancer [43,46]. Heselmeyer-Haddad *et al* showed that extra copies of 3q can be detected in up to 76% of high-grade premalignant lesions and that genomic amplification of *hTR* detected in Pap smears has a predictive value for the development of cervical cancer [47,48]. Concordant with these findings, FISH analysis revealed increased copy numbers of *hTR* in all SCCs and HPV-immortalized cell lines analysed. The present study revealed that a gain of chromosome 3q is more specific for cervical SCC than for AdCA. In contrast to our findings, Yang *et al*, using classical CGH, described a frequent 3q gain in cervical AdCA [23]. The commonly gained region (3q28∼ter) is on the outer telomeric regions of the chromosome, which is often difficult to interpret with classical CGH due to telomere effects [49]. A larger region was found to be gained in only four of the 20 cases (20%) described by Yang *et al*: this is in accordance with our findings.

In the present study, we found low-level gains of 20q in 63% of cervical carcinomas. Although no significant differences were found between the frequency of a 20q gain in SCC and AdCA, extended studies on a larger number of samples will reveal whether such a difference may still exist. A number of previous studies, using classical CGH, describe gains of 20q in 15–30% of cervical cancers [17,22,50,51]. The array CGH used in this study had, in addition to an average resolution of 1 Mb, contigs of BAC clones located at 20q11 and 20q13, which might explain the higher percentage of 20q gains found. In other solid tumours, such as breast, colon, and gastric carcinomas, high-level gains or amplifications of 20q are associated with a selective growth advantage and a more aggressive, metastatic phenotype [52–56]. Low-level gains, on the other hand, have been suggested to be associated with HPV16-E7-induced immortalization of human epithelial cells [57,58]. In support of this suggestion, we detected low-level 20q gains in all of our HPV-immortalized cell lines. Further fine mapping of this region by means of MLPA analysis revealed frequent copy number increases of a number of genes located at 20q11–12, including *DNMT3B* and *TOP1*. Increased copy numbers of the *DNMT3B* gene were

shown to correlate with elevated mRNA expression in 79% of samples analysed.

In summary, we have found significant differences in the chromosomal signatures of cervical SCC and AdCA. Fine mapping of a frequently gained region on 20q yielded a number of potential marker genes for stratification of hr-HPV-positive women at risk for ≥CIN 3. Further evaluation of the frequently found genomic aberrations and expression of genes located within these regions, both in cervical carcinomas and in pre-malignant cervical lesions, is necessary to determine their value as biomarkers for progressive CIN disease.

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