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Human papilloma virus status and chromosomal imbalances in primary cervical carcinomas and tumour cell lines

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Abstract

Human papilloma virus (HPV) infection is the crucial step in the initiation of cervical carcinomas. In addition, HPV18 has been implicated in tumour progression and adverse clinical outcome. We determined the HPV types in 12 primary cervical carcinomas and 12 cell lines and compared the findings with the comparative genetic hybridisation (CGH) pattern of chromosomal alterations. The most frequent alteration was the deletion at 3p14 followed by the loss of 2q34-q36 along with 3q gain. High risk HPV types were detected in all samples except one primary tumour. In contrast to the normal distribution, HPV18 was present in 75% of cases including all cell lines. The cell lines carried a higher number of genetic alterations and a different CGH pattern for several chromosomes than the primary tumours, despite microdissection. Purely HPV18 positive cases indicated a high incidence of imbalances at specific loci with peaks of the histogram coinciding with known HPV integration sites. The study suggests that HPV infection is associated with a recurrent pattern of chromosomal changes in cervical carcinomas and that the development and progression of these alterations is triggered by integration into the host genome. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: CGH; Cervical carcinoma; HPV; Chromosomal imbalances

1. Introduction

Uterine-cervical carcinoma represents the second most common malignancy in women worldwide, both in incidence and mortality. More than 471 000 new cases are diagnosed every year, especially amongst the economically disadvantaged in developing and industrialised nations [1]. In Mexico this neoplasm represents the primary cause of death amongst the female population. Even when there are diverse known factors that might be related to the development of this tumour, including the number of living births, number of sexual partners, first sexual intercourse at an early age and smoking [2], human papilloma virus (HPV) infection is considered to be the initiating event in cervical epithelial transformation [3]. More than 100 HPV types have been identified [4], and high risk HPV types account for more than 80% of all invasive cancers and are already detectable in cervical intra-epithelial neoplasia [5]. HPV infection deregulates cell cycle control via interaction of viral oncoproteins and cellular tumour suppressor proteins, and this phenomenon seems to be critical for cell immortalisation. However, only a fraction of HPV infected patients develop cancer, suggesting that infection, viral oncogene expression and HPV integration must cooperate with a specific genetic background of the host to initiate the multistep process of cervical carcinogenesis [6].

Several studies have addressed this issue using different approaches, including cytogenetics [7], loss of heterozygosity [8] and, more recently, comparative genomic hybridisation (CGH). CGH has led to a model in which gain of chromosome arm 3q is regarded as the specific chromosomal alteration involved in the progression from severe dysplasia to invasive carcinoma of the cervix. Since the 3q gain is usually maintained in advanced tumour stages it represents one of the most prevalent changes associated with disease progression [9–12].

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In the present study, we analysed 12 primary invasive squamous cell carcinomas (SCC) of the cervix together with 12 cervical SCC-derived cell lines by CGH and HPV-typing in order to detect genetic alterations associated with infection status and tumour progression.

2. Material and methods

2.1. Tissue samples

Paraffin-embedded tumour material was collected from hysterectomies performed between 1994 and 1997 at the Institute of Pathology of the University Hospital Charité, Berlin, Germany. Tumours were staged and classified according to the guidelines of the International Union Against Cancer (UICC) and the World Health Organisation (WHO). After diagnosis on a haematoxylin and eosin (H&E) stained slide, defined tumoral tissue areas were microdissected from 25 serial sections each about 5 μm thick, totalling around 125 μm of tissue. Microdissection was performed manually without the use of a microscope. After deparaffinisation, DNA was isolated by proteinase K digestion and phenol-chloroform extraction.

2.2. Cell lines

The 12 cell lines studied were established at the National University of Mexico from IIa and IIb stage tumours derived from 120 tumour explants by standard procedures [13]. The clinicopathological data of the cell lines and the primary tumours is summarised in Table 1.

2.3. HPV detection and typing

HPV infection status was assessed by polymerase chain reaction (PCR), using the consensus primers for the L1 region MY 09 (5′-CGT CCM ARR GGA WAC TGA TC-3′) and MY11 (5′-Biotin-GCM CAG GGW CAT AAY AAT GG-3′, where M = A + C, R = A + G, W = A + T, Y = C + T) [14]. After denaturation at 94°C for 5 min, 40 amplification cycles were performed at 94°C for 1 min, 55°C for 2 min and 72°C for 3 min with a final extension step of 7 min at 72°C. These conditions yield consistently an amplicon of approximately 450 base pairs. The specificity of the PCR products and their typing were established using the hybrid capture method (DIGENE Corp., Beltsville, USA). Briefly, upon hybridisation with a specific single-stranded RNA probe to the 5′-biotinylated PCR product, the specific RNA:DNA hybrids were bound to the surface of streptavidin-coated microwells and detected by an enzyme linked anti-hybrid antibody assay. Two probe mixtures were used in pre-screening experiments to discriminate low and high risk HPVs. The HPV RNA probe A (low risk group) correspond to types 6, 11, 42, 43, and 44 and the probe B (high risk group) to the types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58. The high risk group responsive samples were subsequently examined in an identical way with single RNA probes for the types 16, 18, 31, 33 and 35, respectively. In each case, the DNA quality was checked by amplification of the β-globin genomic sequence. DNA samples of the same extraction were used both for HPV typing and CGH analysis.

2.4. CGH analysis

The protocols for CGH preparation have been described previously and are available on our web site at *http://amba.charite.de/cgh/*. Similarly, image acquisition and digital image analysis were performed as previously described. Briefly, 10 to 15 metaphases/karyograms were analysed per tumour sample with computation of CGH sum-karyograms and mean ratio profiles with confidence intervals [15–17]. Normal reference DNA was derived from peripheral blood leucocytes of female donors. Hybridisations were done on to commercially purchased male metaphase spreads (Vysis). The sex chromosomes were generally excluded from the numerical evaluation of DNA imbalances.

Table 1
Clinicopathological and genetic parameters of the tumour collective

Case	pTNM	FIGO	HPV	Gains	Losses	Σ
G1	pT4 pN1	IVa	None	12	13	25
G2	pT4 pN1	IVa	16	21	13	34
G3	pT1b pN1	Ib	18	10	10	20
G4	pT1b pN0	Ib	16; 18	9	13	22
G5	pT2b pN1	IIb	16	12	11	23
G6	pT1b pN0	Ib	16	6	7	13
G7	pT1b pN0	Ib	18	22	8	30
G8	pT2b pN0	IIb	16; 18	14	12	26
G9	pT1b pN0	Ib	16; 18	8	10	18
G10	pT4 pN0	IVa	18	22	17	39
G11	pT2b pN0	IIb	33	15	7	22
G12	pT1b pN0	Ib	16	9	9	18
T1	pT2s	IIa	18	15	19	34
T2	pT2b	IIb	18	18	14	32
T3	pT2b	IIb	18	17	11	28
T4	pT2b	IIb	18	14	21	35
T5	pT2a	IIa	18	17	11	28
T6	pT2b	IIb	18	13	9	22
T7	pT2a	IIa	18	17	11	28
T8	pT2a	IIa	16; 18	10	10	20
T9	pT2b	IIb	18	23	16	39
T10	pT2b	IIb	18	22	17	39
T11	pT2b	IIb	18	16	11	27
T12	pT2b	IIb	18	10	14	24

HPV, human papilloma virus; G, primary tumours; T, cell lines; FIGO, staging system according to the Fédération Internationale de Gynécologie et d'Obstétrique; Σ, number of changes.

2.5. Determination of chromosomal imbalances

DNA gains and losses were determined by statistical methods, i.e. deviations of the mean FITC:TRITC profiles from the normal ratio of 1.0 were tested for significance by a Student's *t*-test. In this test we required at least a significance of 95% to score an alteration as a DNA gain or loss. After the statistical determination of the chromosomal imbalances of each sample using 95% and 99% significance in the Student's *t*-test, a histogram for all cases was calculated. It included all samples and represented the incidence of DNA gains and losses of the sample group along all chromosomes, showing gains at the right of the ideogram and losses at the left of the ideogram, e.g. the maximum value of 100% is reached if all samples of the group carry an imbalance at a specific chromosomal region. The two significances are indicated by distinct colours: blue areas indicate those alterations with 99% significance whereas the green areas include those with 95% significance. Pronounced DNA gains and losses were defined as those alterations for which the ratio profiles exceeded the values of 1.5 and 0.5, respectively. They most likely represent high copy number amplifications or multi-copy deletions. In Figs. 1 and 2 they are shown in red.

3. Results

The histopathological data of the primary tumours and cell lines, together with the HPV status and the numerical analysis of the CGH findings are summarised in Table 1.

3.1. HPV genotyping

DNA from the microdissected areas was subjected to PCR amplification for HPV genotyping. In the primary tumours HPV16 was detected in 4 of 12 cases, HPV18 in 3, HPV16/18 coinfection in 3, HPV33 in one sample and only one tumour did not carry the HPV sequences. All the cell lines presented HPV18, in one case with coinfection of HPV16. As a control of the DNA quality for PCR, all samples were successfully amplified for the β-globin genomic sequence.

3.2. CGH analysis

The results of the primary tumours and cell lines are summarised in Tables 1 and 2. Individual ratio profiles can be viewed and combined to CGH histograms on our homepage *http://amba.charite.de/cgh/*. On average,

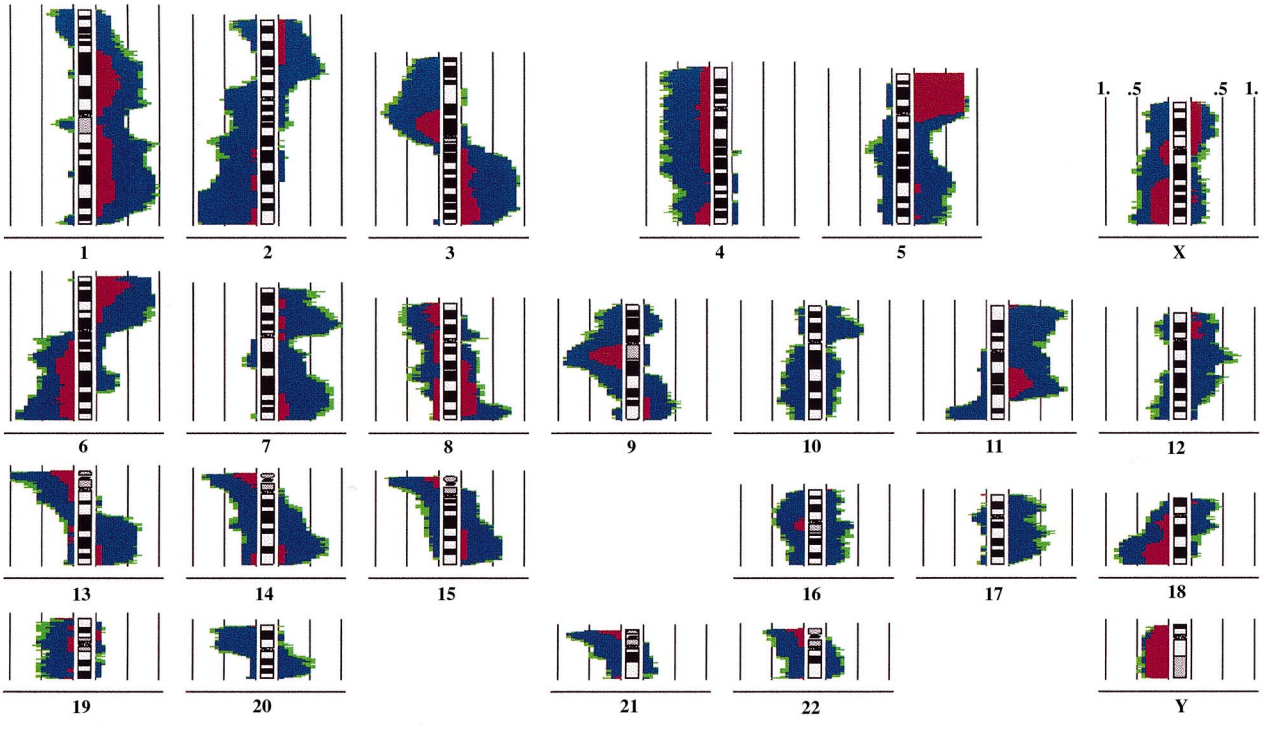

Fig. 1. Summary of the genetic alterations of 24 cervical carcinomas, i.e. 12 primary tumours and 12 tumour cell lines, in a histogram presentation. Areas on the left side of the corresponding chromosome ideogram demonstrate the relative number of cases with DNA losses, whereas overrepresentations of genetic material in the samples are shown to the right of the ideograms. The percentage of cases with changes that are significant with a confidence of 99% are shown in blue, the percentage of cases with alterations of confidence between 95% and 99% are shown in green, regions where the imbalance exceeded the 1.5 and 0.5 threshold are shown in red. Heterochromatic areas (centromeric and paracentromeric regions of chromosomes 1, 9, 16, p arms of acrocentric chromosomes) were excluded from the analysis.

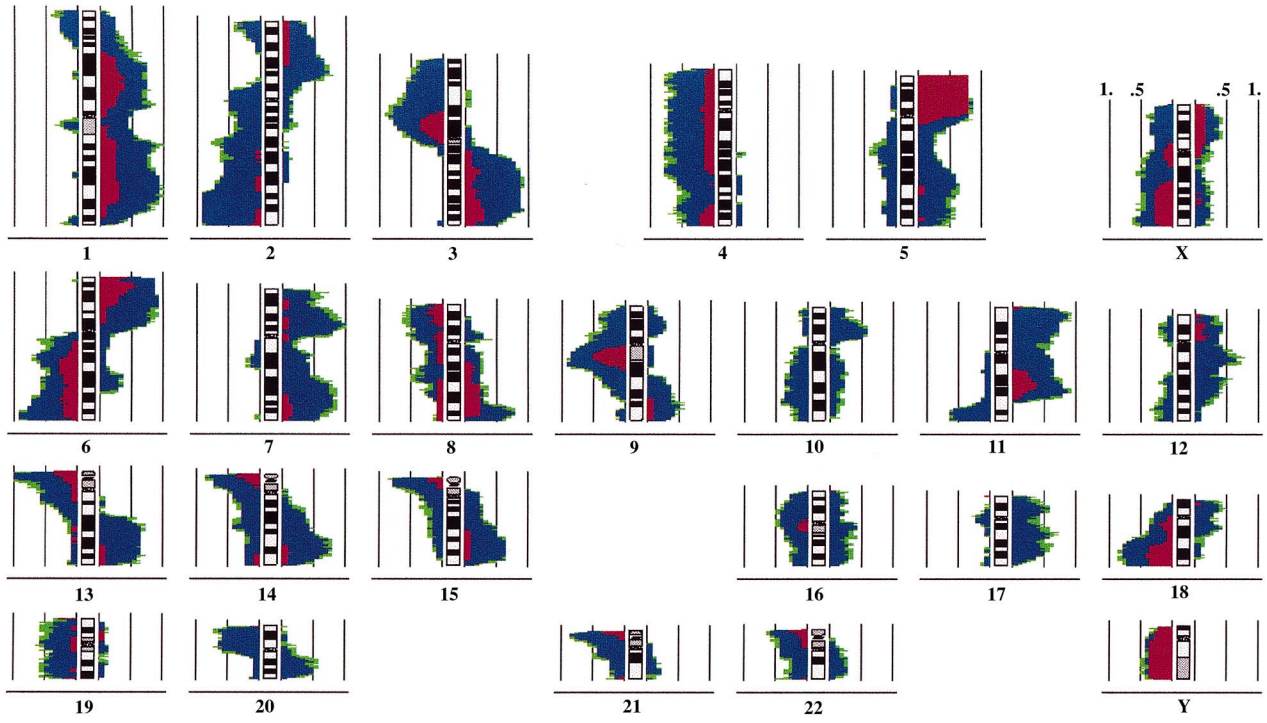

Fig. 2. Histogram of all genetic alterations of the purely HPV18 positive cases including 14 cases, i.e. 3 primary tumours and 11 cell lines. Representation of DNA gains and losses, as well as the confidence intervals are the same as in Fig. 1. Interestingly, some chromosomal alterations are present in more than 85% of the cases, suggesting a possible relationship between these alterations and HPV18 infection.

our tumour collective carried 27 aberrations per case, i.e. 15 gains and 12 losses. Cell lines presented a higher number of genetic imbalances ($\Sigma = 30$) than the primary tumours ($\Sigma = 24$). All cases presented alterations and samples with advanced tumour stages (for the most part) showed a higher number of aberrations.

The CGH histogram of all 24 cases, representing the incidence of chromosomal imbalances determined by statistical analysis is shown in Fig. 1. The highest frequency of alterations was observed at chromosome 3p14 being present in up to 20 cases (83%) followed by gain at 3q and loss at 2q34-q36 in 19 cases (79%). Additional alterations with at least 30% incidence are listed in Table 2.

Generally, the pattern of imbalances for the majority of the chromosomes was similar between the primary

Table 2
Chromosomal imbalances in squamous cell carcinomas of the cervix

	DNA underrepresentations	DNA overrepresentations
Primary tumours ($n = 12$)		
High incidence changes ($\geqslant 50\%$)	2q22-q23, 2q32-qter, 3p, 4, 5q14-q23, 5q32-q33, 6q12-qter, 11q23-qter, 13q21-q32.	1p21-p22, 1p31-pter, 1q21-q24, 3q, 14q21-q23, 16p11.1-p13.2, 16q12.2, 16q22-q23, 17q21-qter, 19, 20q11.1-q13.2, 22q12-q13
Less prevalent changes ($> 30\%$, $< 50\%$)	2q22-q32, 7q32-qter, 11q14-q23, 13q32-qter, 18q22	1p11-p13, 1p22-p31, 1q25-q42, 2p11.2-p22, 5p, 7p13, 7q11.2-q11.3, 8q, 9p21, 9q22, 11p11.1-p13, 12q13, 14q24-q31, 15q21-q26
Pronounced changes[a]	3p25-p26, 6q14-q26, 11q21-qter, 18q22	3q22-q23, 3q26.1-qter, 5p12-p15, 11q14-q22, 19p13.1-p13.2
Cell lines ($n = 12$)		
High incidence changes ($\geqslant 50\%$)	2q, 3p11-p21, 4, 6q, 8p, 11q24-qter, 14q22, 16p11.2-p13.1, 18q12-qter, 19, 20p	1p13-p31, 1q12-q42, 2p14-p21, 3q13.2-qter, 5p, 5q21-q34, 6p, 7p12-pter, 7q21-qter, 8q24.1-qter, 10p11.2-p12, 11p12-pter, 11q12-q22, 12q12-q13, 13q21-qter, 14q24-q32, 15q24-qter, 17p12, 17q21, 20q13.1-q13.3.
Less prevalent changes ($> 30\%$, $< 50\%$)	1p35-pter, 2p25-pter, 3p21-p24, 8q21.1-q21.3, 9p13-p23, 10q21-q25, 14q12-q21, 16q11.2-q21	1q43-qter, 5q21, 6q22-q23, 8q23, 14q23, 17q12-q24
Pronounced changes[a]	3p11-p14, 4p11-q26, 4q32-qter, 6q16-qter, 8p21-p23, 18q11, 18q21-qter	1p13-p31, 1q21-q41, 3q22, 3q26.1-qter, 5p11-pter, 8q22-q24.3, 11q14-q22, 12p11-p13, 14q31
HPV18 tumours ($n = 14$)		
$> 90\%$ incidence	2q33-qter, 3p12-p14, 6q27	1q31-q32, 3q22-q26.3, 5p14, 6p21.3-p22, 7p13-p14

[a] DNA gain with ratio > 1.5, DNA loss with ratio < 0.5, only those alterations which occurred at least twice were listed.

tumours and cell lines. The cell lines, however, carried a higher proportion of pronounced gains in particular, but also pronounced losses, indicating high copy DNA amplifications and multi-copy deletions as summarised in Table 2. They also showed more extensive deletions, e.g. for chromosomes 2q, 8p and 18q. In addition, there were notable differences for several chromosomes, particularly for 3p, 5, 6p, 7, 11, 13, 16, 19 and 20.

For chromosome 3p, the primary tumours showed more deletions of the telomeric part whereas the cell lines harboured more underrepresentations of the centromeric part. For chromosome 5, the short arm was amplified in most cell lines whereas, unlike in the primary tumours, 5q was rarely lost. Similarly, chromosomes 6p and 7 carried more amplifications and overrepresentations in the cell lines. Deletions of the telomeric part of chromosome 11 was higher in the primary tumours, whilst the cell lines presented more amplifications of 11p and also the centromeric part of 11q. Chromosome 13 including the telomeric part 13q21-qter was more frequently lost in the primary tumour cases, whilst the cell lines presented more amplifications at the 13q21-24 region. There was a high proportion of gains in chromosome 16 in the primary tumours, whereas losses predominated in the cell lines. Whilst primary tumours showed chromosome 19 gains, cell lines presented more often losses of this chromosome. Deletions on 20p were observed exclusively in the cell lines.

A histogram of all the purely HPV18 positive cases, representing 11 cell lines and 3 primary tumours is presented in Fig. 2. We observed an incidence of more than 90% for the DNA gains at 1q31-q32, 3q22-q26.3, 5p14, 6p21.3-p22 and 7p13-p14, as well as the DNA losses at 2q33-qter, 3p12-p14 and 6q27. In addition, the histogram carried several peaks of the incidence curve, e.g. at 8q24.2, 12q13 and 18q21-q22.

4. Discussion

Uterine cervical carcinoma is characterised by a recurrent pattern of chromosomal imbalances [9–12], however, their importance in tumour progression and their relation to HPV types has not been fully explored.

4.1. HPV and chromosomal alterations

In this study we observed a high incidence of chromosomal imbalances at specific chromosomal regions in the HPV18 positive cases. All these loci have been described as HPV integration sites or fragile sites within the genome [18–22]. Specifically, the 7p13-p14 region has been shown to harbour a transcribed human sequence related to HPV18 *E5* gene, named pe5l, suggesting that pe5l is a target for HPV recombination and integration [23]. HPV18 integration sites in the HeLa cell line are colocalised with c-myc amplicons at 8q24 and throughout the cell's genome, indicating that integration preceded amplification [24]. Other recurrent chromosomal alterations, including specific deletions and amplifications have also been observed near HPV integration points [25]. HPV immortalised cells have already developed several specific chromosomal aberrations, some of which have also been detected in HPV positive tumour samples [26–28]. Incidence studies around the world indicate that HPV16 is present in 50% of the tumours, followed by HPV18 (14%) [5]. The primary tumours of our study were derived from German patients and presented with a similar HPV distribution. However, the cell lines derived from Mexican patients did not present this expected pattern. This situation cannot be explained by a higher prevalence of HPV18 in this population since HPV type distribution in Mexico is not significantly different from the rest of the world [29]. Studies in Mexico indicate that HPV18 is preferentially observed in invasive cervical carcinoma cases [30]. Similarly, HPV18 positive tumours have been associated with a worse prognosis in contrast to other HPV types [31]. This is a possible explanation for the observed prevalence of HPV18 in the cell lines, since cell cultures can generally be established more easily from aggressive primary tumours and their genetic alterations are known to reflect those that are important for tumour progression. Interestingly, prevalent changes of HPV18 positive tumours like the DNA gains on 1q31-q32, 8q24 and deletions on 3p12-p14, 4pter, 6qter correlate with those alterations which we previously found to be significantly associated with the metastatic phenotype of lung SCC providing a putative explanation for the worse prognosis of HPV18 tumours [32].

4.2. Incidence of DNA imbalances in cervical carcinomas

There is no consensus yet on the best way to determine DNA changes by CGH. Many laboratories prefer fixed thresholds for scoring DNA gains and losses often using different values for DNA samples derived from archival tissue (formalin-fixed, paraffin-embedded) and viable cells. We used instead a statistical procedure which is more sensitive for scoring chromosomal imbalances than frequently used fixed thresholds like 0.85 and 1.15 (16, 32, data not shown). The reasons for this are manifold. Fixed thresholds are still arbitrary although they may have resulted from control experiments; the quality of the hybridisation and thus the fluorescence signal used for digital image analysis is influenced by many factors of which formalin-fixation and paraffin-embedding are only two. There are samples from archival tissue with excellent signals whereas high quality DNA samples may also give poor results. Thus,

in our view, it is not justified to generally apply distinct threshold values for different kinds of DNA samples; there is considerable evidence recently that statistical evaluation is more accurate and thus, the method of choice [33,34]. The histogram of Fig. 1 was calculated by scoring significant deviations of the ratio profile from the normal value 1.0 according to the Student's *t*-test using 95 and 99% significance levels. It is based on the analysis of all 24 cervical carcinomas and indicates the incidence of DNA gains and losses at specific chromosomal sites providing an overview of those regions that are characteristically affected in this tumour. In our view, the histogram representation is the most appropriate way to visualise the chromosomal imbalances of large tumour collectives. Even the results of several hundred cases can be demonstrated which is impossible by a line representation. In contrast to sum ratio profiles, the histogram provides much richer information on the incidence of an imbalance at a particular chromosomal site and also allows the representation of pronounced DNA gains and losses correlating with high copy amplifications or multicopy deletions. Both, sum profiles and line representations which are still useful for small collectives can be viewed at our CGH online tumour database web site. Histograms can also be calculated by using fixed ratio thresholds. Since we recently implemented the final part of our custom-made CGH software (the so-called CGH-Super Program for the calculation of histograms, sum ratio profiles, line representations, difference histograms, etc.) in Java, any reader may visualise and explore our data via the Internet at *http://amba.charite.de/cgh/*. Importantly the same data set can be analysed by the different evaluation schemes that are currently used for determining chromosomal imbalances by CGH, i.e. statistical methods and fixed thresholds. These thresholds can be adjusted offering each reader/user the possibility to compare our results with their own using their personal method of evaluation.

The increased sensitivity of our method does correlate with the higher incidence of imbalances and, in particular, of deletions that we observed in this study compared with previous ones [9–12]. Our previous studies showed an excellent agreement of CGH findings when either control experiments like inverse labelling or additional molecular genetic tests were applied (e.g. 15, 17). Therefore, we believe in the validity of the present data even though no additional analysis was applied.

The alteration with the highest incidence was the deletion at chromosome 3p14. This is in agreement with earlier studies by allelotyping, pointing to the importance of this chromosome region in the very early stages of tumorigenesis [8].

Other studies reported that the 3q gain is present in almost all carcinomas (90%), although it was reported in only 1 of 13 severe dysplasia [9]. Investigations of dysplasia alone revealed no gain on chromosome 3q [11]. This has led to a model in which the 3q gain is considered the key alteration in the transition to invasive carcinomas. However, our results suggest that the deletion on chromosome 2q34-q36 is as important as the 3q gain since it occurred with the same frequency.

4.3. Comparison of primary tumours and cell lines

We observed a higher number of alterations in the cell lines compared with the primary tumours. This might be explained by several reasons. Primary carcinomas are often a mixture of tumour tissue, inflammatory cells and normal stroma thus, decreasing the percentage of tumour cells compared with pure cell lines. However, the influence of normal cell contamination was largely excluded by microdissection. In addition, intratumoral genetic heterogeneity might also decrease the sensitivity for detecting certain alterations. Since the primary tumours exhibited a considerable variability of HPV types and also a higher frequency of viral coinfections we feel that tumour heterogeneity is indeed more pronounced in the primary tumours compared with the cell lines and may thus, reduce the sensitivity of the detection of DNA imbalances. Moreover, CGH analysis using formalin-fixed, paraffin-embedded tissue might yield less intense fluorescence signals than DNA derived from viable cells or from fresh frozen tissue. However, since we evaluated only high quality hybridisations from formalin-fixed samples and also applied a very sensitive method for the determination of chromosomal alterations, we are convinced that this factor did not have a significant influence on the number of chromosomal changes. As mentioned above, cell lines generally represent more advanced tumour stages which carry more genetic changes than early stage tumours. Finally, cell lines may also select for and accumulate additional genetic changes during cell culturing.

For several chromosomes we found a different CGH pattern in both tumour types. We observed more extended deletions and smaller size amplifications in the cell lines compared with the primary tumours. A similar pattern was observed during the progression of lung squamous cell carcinomas to a metastatic stage, again corroborating the observation that cell lines generally represent more aggressive tumours [32].

In summary, this study further supports the evidence that cervical carcinomas are characterised by a recurrent pattern of chromosomal alterations with specific imbalances being important for tumour initiation and progression. In addition, it supports the assumption that these chromosomal changes are triggered by the integration event suggesting that HPV integration is a necessary step for tumour initiation. Finally, it points to the possible association between specific HPV types and distinct genetic lesions.

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