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ARTICLE

Chromosomal Aberrations Accumulate in Polyploid cells of High-grade Squamous Intraepithelial Lesions (HSIL)

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Persistant infection with human papillomavirus (HPV) of the uterine cervix is related with cytological atypia (SIL), the oncogenic potential of which is unclear in a given time point of monitoring. HPV-induced genetic instability result in polyploidization as well as in low frequency random chromosome aberrations in squamous cells. In the present work we analyzed whether highly polyploid/aneuploid cells reflect genomic changes at the chromosomal level. 13 samples with the cytological diagnosis of HSIL were analyzed for HPV type and nuclear DNA content measured by laser scanning cytometry (LSC). Hyperdiploid cells with >5c and with >9c DNA content were further analyzed for numerical aberrations of the chromosomes 3 and 17 by fluorescence in situ hybridization (FISH) following repositioning. Cells with >5c DNA content were found more frequently than cells with >9c DNA content (5-98 and 1-44 cells, respectively). The FISH analysis demonstrated frequent polysomies, however, the rate of aneusomy (other than 2, 4, 8 or 16 chromosome copies) was significantly higher in cells with >9c DNA content than in cells with >5c DNA content or the normal diploid cells. The imbalance of chromosome 3 and 17 copy number was also increased in cells with >9c DNA content. Moreover, in three out of the 13 analyzed HSIL samples, recurrent abnormal chromosome 3/17 ratio was demonstrated in a significant part of the cells, indicating a common origin of these cells. Highly polyploid/aneuploid cells in HSIL accumulate cytogenetic aberrations detectable by FISH analysis. These cells may reflect early changes with tumorigenic potential in a very concentrated fashion. (Pathology Oncology Research Vol 10, No 3, 142–148)

Keywords: HPV, aneuploidy, chromosome, cytology, cervix

Introduction

Infection by high-risk human papillomavirus (HPV) was reported to be strongly associated with the evolution of cervical cancer, however, the genital infection is spontaneously eliminated within several months in the majority of the cases.[1,2,3] Persistant HPV infection is characterized by marked cytological atypia. Squamous intraepithelial lesions (SIL) occur as morphological representants of HPV-related biological and genomic dysfunction.[4] The lesions are classified as low-grade and high-grade SIL according to the degree of atypia and the risk to develop cancer by The Bethesda Systems for Reporting Cervical/Vaginal Cytologic Diagnoses.[5,6] Unfortunately, the morphological changes are of low specificity to predict the outcome in the early phase of the disease. Nuclear DNA content aberrations have been suggested as characteristic for tumorigenic lesions as a significant portion of cervical cancers proved to be aneuploid.[7,8] The effectiveness of DNA ploidy analysis in early precancerous lesions is, on the other hand, limited by the low number of dysplastic cells and the occurrence of a wide range of random genetic aberrations. The picture is further complicated by the fact that HPV facilitates polyploidization in squamous

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cells. This was demonstrated for both low-risk and oncogenic types of HPV,[9,10] therefore polyploidization could not be directly associated with malignant transformation so far. Only stem-line DNA-aneuploidy and the demonstration of cells with a DNA content exceeding 5c or even 9c were emphasized to characterize high-grade lesions.[11]

In a previous study we found a direct correlation between the occurrence of squamous cells with larger than >9c DNA content and high-risk HPV infection in "atypical squamous cell of undetermined significance (ASCUS)" and "high grade SIL (HSIL)".[12,13] Here, laser scanning cytometry (LSC) was used to search for rare aneuploid cells in routine cervical cytological samples.[14] Cells were gated on the basis of their total nuclear fluorescence following DNA staining and could be repositioned for morphological inspection. LSC also supports the in situ analysis of individual aberrant cells for genomic changes by fluorescence in situ hybridization (FISH) following DNA cytometry. In order to investigate high-risk HPV induced highly polyploid cells regarding genomic changes at the chromosomal level, copy numbers of chromosomes 3 and 17 were analyzed in detail by FISH in cells gated on the basis of the nuclear DNA content by LSC. By the use of centromere-specific DNA probes we demonstrated polysomies, aneusomies, and apparently more complex genomic aberrations in polyploid cells of the cervical squamous intraepithelial lesions.

Materials and Methods

Cytological diagnosis

Altogether 13 samples with the cytological diagnosis of high-grade "squamous intraepithelial lesions (SIL)" according to The Bethesda System[6] were selected because of a high frequency of cells with abnormally high DNA content. The samples were analyzed for DNA ploidy, chromosomal changes and HPV positivity. Routine liquid fixed (CytoLyt, Cytyc, Boston, MA) cervical cytological samples were used for all purposes. For routine cytology and DNA content measurements cell monolayers (ThinPrep, Cytyc) were prepared according to the manufacturer's protocol. Cytological diagnosis was made following the conventional Papanicolaou staining and the diagnosis of HSIL was made using criteria of The Bethesda System.

DNA cytometry

DNA content measurements were performed on a second monolayer of the same material which was stained in a solution containing 50 μg/ml propidium iodide (PI) and 200 μg/ml RNase at 37°C for 1 hour. The cells were covered with glycerol/PI (25 μg/ml) and a coverglass. Automated PI-related fluorescence measurements of whole monolayer preparations were performed in a laser scanning cytometer (LSC, CompuCyte, Boston, MA). Normal (2c) DNA content was determined according to the first peak intensity value of the DNA histogram, which contained normal leukocytes stated by eye control. Coefficients of variation (CV) of the DNA-histograms were between 4.0 and 7.5. Events with > 5c and >9c DNA content were gated and individually evaluated in the microscope of LSC following computer assisted repositioning. Artifacts and occasionally occurring cell doublets/conglomerates were excluded from the analysis. "Rare cells with abnormally high DNA content" according to the ESACP Consensus Report,[15] which were isolated cells with an atypical cell morphology and a DNA content of >5c or >9c were further analyzed by FISH.

Fluorescence in situ hybridization

Cells selected by gating larger than 5c and 9c DNA content were analyzed for chromosome 3 and 17 numerical changes by FISH following the measurement of the DNA content. For this purpose, coverglasses were detached in phosphate buffered saline (PBS) and the slides were postfixed in a 3:1 mixture of methanol and glacial acid (10 minutes, -20°C). Following air drying, cell-containing areas were exposed to pepsin digestion (0.5% end concentration, pH 1.5) for 5 minutes at 37°C and the proteolysis was stopped in 3.6% formaldehyde/PBS following rinsing the slides in PBS. After another wash step in PBS for 5 minutes, the preparations were dehydrated through an ascending alcohol series and air dried. For FISH, direct fluorescence-labeled chromosome 3 (SpectrumOrange) and chromosome 17 (SpectrumGreen) alpha-satellite DNA probes were applied to the slides according to the manufacturer's description (Vysis Inc., Dovers Grove, IL). Following the posthybridization washing the slides were fixed in 3.6% formaldehyde/PBS for 5 minutes at room temperature, dehydrated in ethanol and covered by an antifade medium containing the DNA stain DAPI (Vectashield, Vector Corp., Burlingame, CA). Repositioning of cells with aberrant DNA-content was done in the LSC and each cell was analyzed for red and green fluorescence hybridization signals. Fluorescence images were captured by ISIS digital image analysis workstation (MetaSystems, Altlussheim, Germany) attached to the microscope of the LSC.

Detection and typing of HPV

10 ml of the liquid fixed cytological sample were centrifuged at 2000 g and the pellet was dissolved in 200 μl PBS. DNA was extracted using the DNA Mini Kit (QIAGEN, Hilden Germany) according to the manufacturer's instructions.

In order to detect HPV DNA a two tier PCR-DS method was performed according to Feoli-Fonseca et al[16] with

modifications. We used the general consensus primers GP5+/GP6+[17] and the MY09/MY11[18] primers for amplification of HPV DNA. Reaction parameters were the following: 40 cycles of amplification were run with an initial denaturation at 95ºC for 5 minutes and for 30 seconds in each cycle. 37ºC and 58ºC was set for 30 seconds for annealing of the primer pairs GP5+/GP6+ and MY09/11 respectively, extension was done at 72ºC for 30 seconds. A final extension step was done at 72ºC for 5 minutes. The integrity of human genomic DNA was verified by PCR amplification of the β-globin gene. This reaction served as a positive control. The amplification products of the two consensus primer pairs and the β-globin PCR were run on a 2% agarose gel and stained with ethidium bromide.

PCR products were purified using the High Pure PCR product purification Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The sequence of one strand of the purified PCR fragments was determined with the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA) using 3-5 pmol of GP5+ or MY09 as the sequencing primers. The results of the sequencing reactions were analyzed on an ABI Prism 310 automated sequencer (Applied Biosystems).

The obtained sequences were compared with documented virus sequences available in the GenBank databank using the BLAST program (Blast, Pittsboro, NC).

Results

Frequency of cells with aberrant DNA content in HSIL in relation to HPV type

In this study, 13 HSIL samples presenting with >9c events were selected for further evaluation. The specific features of these cases are given in *Table 1*. All samples proved to be infected with a high-risk type HPV. The average number of cells analyzed for DNA content was 59,467 per slide, with a range between 42,800 (case no. 5) and 72,666 (case no. 3). Isolated cells were all characterized by an individual DNA value due to PI fluorescence intensity. These values were displayed as a DNA histogram, where 5c and 9c regions could be gated. The frequency of cells with abnormally high DNA content larger than 5c and 9c was in the range of 5-98 and 1-44, respectively. The number of >5c cells was always higher than that of the >9c cells (*Table 1*).

Aberrant DNA content and polysomy of #3 and #17

All cells from the larger than 5c and 9c DNA content gate were repositioned in the cytometer after performing dual color FISH with DNA probes specific to chromosome 3 and 17. For comparison, 200 cells, randomly selected unrelated to the search result, were also evaluated for chromosome 3 and 17 signals.

Table 1. **HPV type, number of polyploid cells detected by LSC and short-term follow-up in 13 samples with HSIL**

		Number of cells exceeding		
Sample	*HPV type*	5c	9c	*Follow-up**
1	16	9	5	CIN III
2	16	30	13	CIN II
3	16	86	19	CIS
4	16	98	44	not available
5	16	41	4	CIN III
6	33	70	30	not available
7	51	5	4	CIN II
8	51	42	14	CIN II
9	51	69	34	CIN III
10	52	25	4	CIN I
11	58	66	4	CIS
12	62	16	1	regression
13	73	64	9	CIN I

*Short-term outcome is based on histological data available after 12 months from the HSIL diagnosis.

In cells of the latter control group, basically two hybridization signals were visible, representing two homologue chromosomes. Small deviations, never exceeding 10% of the total analyzed cell populations, were also observed due to FISH-related inaccuracy. As expected, a significant part of the 5c and of the 9c cells showed polysomies (4, 8 or 16 copies) for both chromosome 3 and 17 (*Figure 1*). This was present in all analyzed samples, however, the frequency of the polysomies was in a wide range (*Figure 2*). The number of cells with polysomy of chromosomes 3 and 17 correlated well.

Aneusomy of chromosome 3 and 17 in polyploid cells

The frequency of cells with FISH spots for chromosome 3 or 17, which do not correspond to a polysomy (other than 2, 4, 8 or 16 copies) in relation to the total DNA-content is demonstrated in *Figure 3*. Aneusomy of both of the analyzed chromosomes was significantly more frequent in 5c cells and further increased in 9c cells in contrast to control cells from the near-to-normal DNA content range ($p<0.005$). Samples with high proportions of polysomy showed low levels of aneusomy and vice versa. In three of the analyzed cases aberrant FISH pattern indicating aneusomy for chromosome 3 was found in more than 70% of the 9c cells. A similar accumulation of aneusomy 17 was also observed in 9c cells *(Figure 3b)*.

Chromosomal imbalance in polyploid cells

To demonstrate genomic instability at the chromosomal level in general we determined the frequency of cells with an imbalanced chromosome 3 and 17 ratio for each DNA

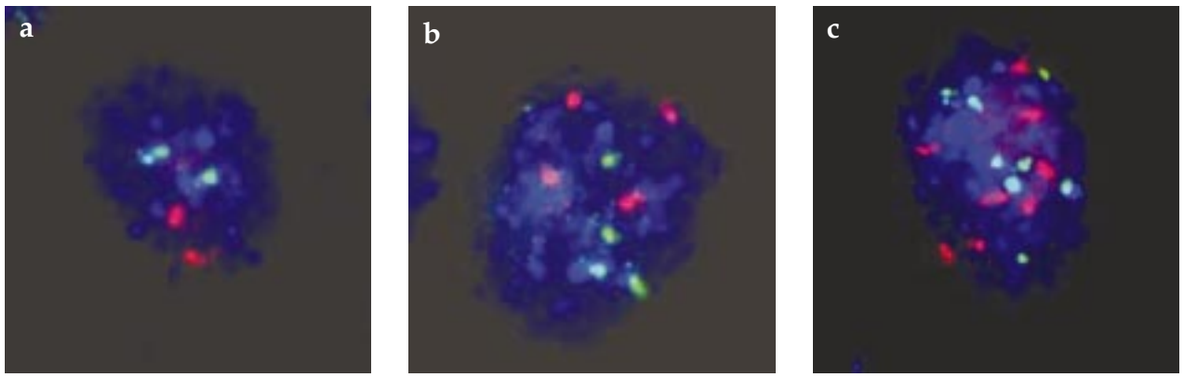

Figure 1. *Fluorescence in situ hybridization with pericentromeric DNA probes specific for chromosome 3 (red) and 17 (green signals) demonstrate balanced polysomy of both chromosomes in cells with elevated DNA content. DNA content (DI) measured by laser scanning cytometry for the depicted cells prior FISH was in* ***a:*** *diploid (DI: 1.03),* ***b:*** *tetraploid (DI: 2.64) and* ***c:*** *octaploid (DI: 7.40) range.*

content range. Similar to individual aneusomies, the frequency of cells showing numerical change for any of the analyzed chromosomes increased significantly with elevated DNA contents ($p<0.001$). The frequency of 3/17 imbalance was up to 92.2%, 41.3% and 17.5% in the >9c, 5c-9c and <5c DNA content range, respectively *(Figure 4).*

Clonal chromosomal aberrations in HSIL

The enumeration of the cells with 3 and 17 aberration enabled us to determine the most frequent type of 3/17 FISH pattern in individual cells. Surprisingly, in three of the analyzed samples (23.1%) a high frequency of a given pattern was observed, suggesting a common origin of these aberrant cells. In sample no. 6, 46.6% of the cells with >9c DNA content showed the same FISH signal ratio of 4/5, representing four copies of chromosome 3 and five copies of chromosome 17 in these cell nuclei *(Figure 5,* top line). In sample no. 9, 52.9% of the cell nuclei displayed a chromosome ratio of 3/4 *(Figure 5,* middle line). An interesting FISH pattern was observed in 5/9 (55.5%) of the gated cells of the sample no. 13 *(Figure 5,* bottom line). While there were only 2 large FISH signals for chromosome 17 in these cells, chromosome 3 signals could be distincted as large and small spots. In addition to two or three large hybridization signals, 4-14 additional small red signals per nucleus was visible resembling to a chromosome locus amplification. A highly similar fluorescence pattern for chromosome 3 were also visible in 12/64 cells (18.7%) of the 5c-9c DNA content gate.

Discussion

Among many other features, the polyploidization of squamous cells seems to be a direct effect of HPV by inhibiting the formation of the mitotic apparatus in the prometaphase of the cell cycle. Centrosome disturbances,

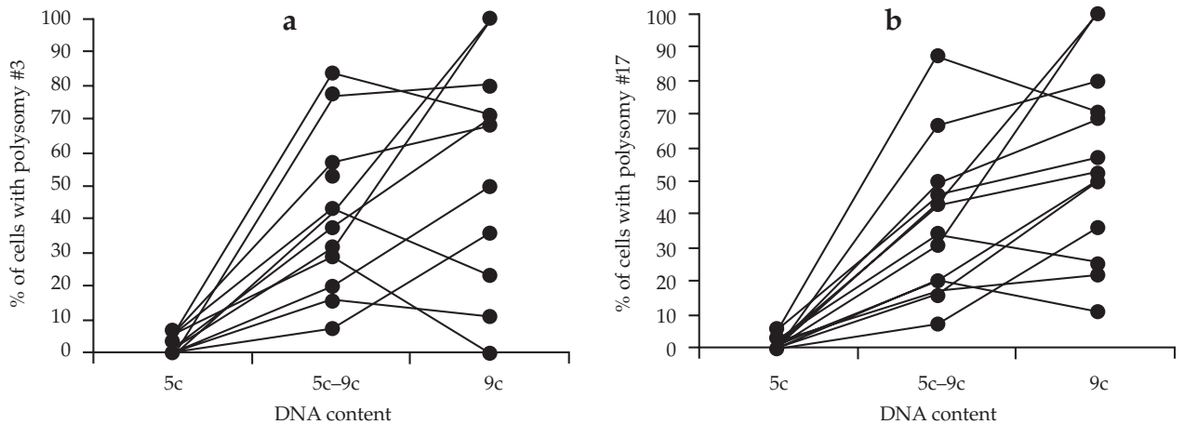

Figure 2. *Frequency of cells with >5c and >9c DNA content showing polysomy of chromosome 3* ***(a)*** *and 17* ***(b)****. Values in the different DNA ploidy categories belonging to the same samples are connected by lines*

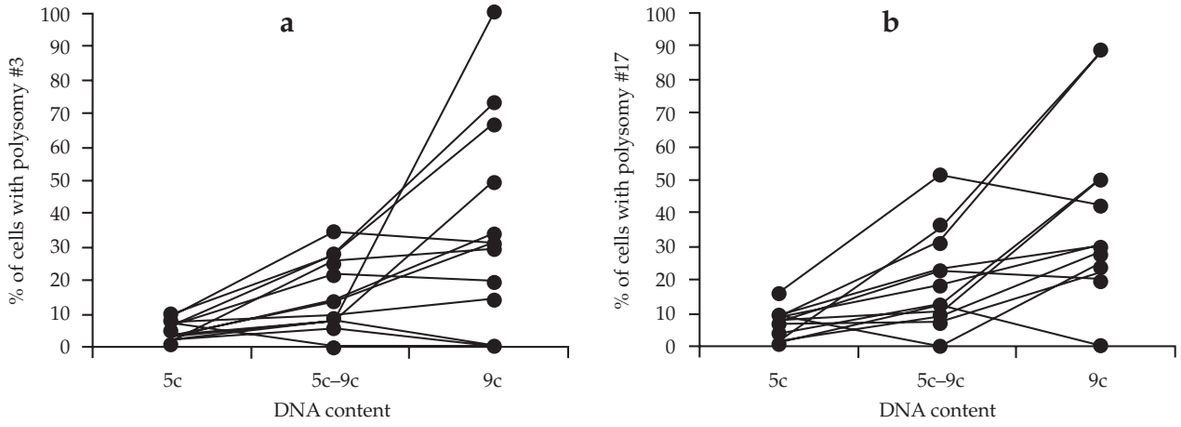

Figure 3. *Accumulation of cells with chromosome 3* ***(a)*** *or 17* ***(b)*** *aneusomy in polyploid cells showing DNA content exceeding 5c and 9c*

occurring in the presence of episomal virus genome have been described as a possible mechanism of endoreduplication.[19,20] The latter leads to the formation of true polyploid cells by duplication of the normal nuclear DNA content (4c) and then further (8c, 16c, etc.), a phenomenon which was described in low- as well as in high-grade squamous intraepithelial lesions.[21,22] However, the same mechanism may induce endoreduplication in squamous cells with preexisting genetic aberrations including aneusomies or structural chromosomal aberrations. Thus, cells with highly elevated DNA content evolve, representing genomic changes of peridiploid cells. Moreover, if the generation of such "polyploid aneuploid" cells is continuous, they also should reflect low frequency clonal aberrations in the initial phase of the disease evolution, when other methods still fail to demonstrate them. To enlight this possibility, chromosome-specific FISH analysis was performed on polyploid squamous cells selected in HSIL. The analysis of numerical chromosomal aberrations relied on the quantification of FISH signals following hybridization with pericentromeric alpha-satellite probes.[23] The DNA content of the cells was determined by laser scanning cytometry prior to FISH. This method enabled us to correlate cell morphology and the quantity of individual chromosomes with the total nuclear DNA content in selected cells. Despite technical limitations originating from the slide based methodology (overlapping of cells and/or microorganisms, incomplete RNase digestion and PI staining, degradation/fragmentation of DNA, autofluorescence in monolayers, etc.), LSC provide automated DNA content measurements of high cell numbers with a reasonable accuracy.[24] This was also reproducible during the present experiments and FISH spot counts also clearly supported the increase in DNA content measured by LSC.

Chromosomal aberrations in cervical cancer have been extensively characterized by both classical and molecular cytogenetics.[25,26] Recurrent structural and numerical aberrations including the frequent involvement of the chromosomes 3 and 17 have been reported in squamous cell carcinoma of the cervix.[27,28] A direct link between integrated viral genome and chromosomal alteration has also been suggested.[29] On the other hand, molecular cytogenetic methods in cervical dysplasia reflected mostly random chromosomal alterations, so far without an evidence of a clonal progression.[30,31]

Our data also clearly showed that polyploidization (cells exceeding 5c and 9c) was in agreement with the multiplication of chromosomes 3 and 17. In many examples only a small number of cells exceeding 9c DNA content was seen, which was always less then the number of 5c exceeding cells. This feature is not surprising if we consider that cells must pass through the 5c range to reach 9c or higher DNA content. However, in addition to polysomy of chromosome 3 and 17, the frequency of random aneusomies of

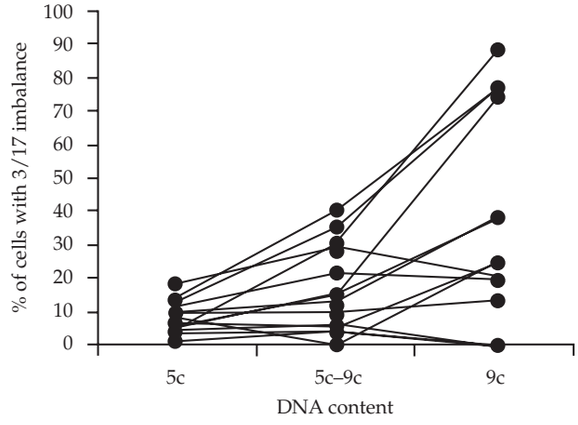

Figure 4. *Frequency of cells with imbalanced alteration of chromosome 3 or 17 copy number. Imbalance of chromosome 3/17 was used as a measure of genetic instability of polyploid cells in HSIL*

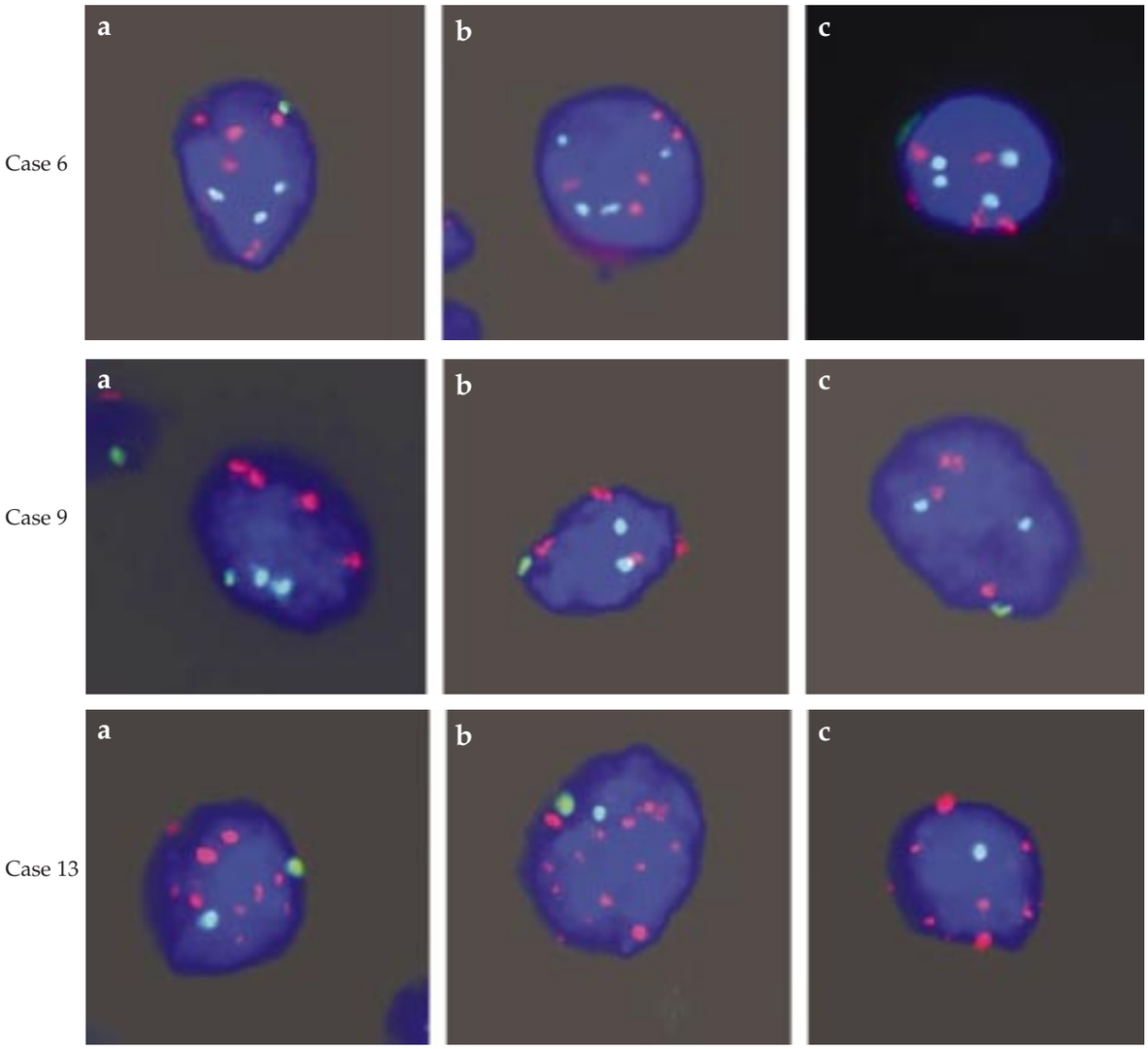

Figure 5. *Recurring imbalance of chromosome 3 and 17 in cells selected by DNA content exceeding 9c. 3/13 samples showed the same chromosome ratio in half of the analyzed cells. Chromosome 3/17 ratio was 4/5* ***(a)****, 3/4* ***(b)*** *and 4-14/2* ***(c)*** *in the samples no 6, 9 and 13, respectively*

the analyzed chromosomes was also obvious in cells with increased DNA content.

According to the data available, cervical cancer stemlines do not show DNA contents in the highly polyploid range. Polyploidization is not associated with clonal expansion and, therefore, the process seems to become a „dead end" for the affected cells. On the other hand, these cells were found to be characteristic for HSIL and could be related to high-risk HPV types. In agreement with our morphological observations, the gating on events exceeding 9c enrich the most outstanding cells of the cytological sample. These cells significantly contribute to the dysplastic appearance and are obvious consequences of HPV infection. Provided that the polyploidization was initiated following the occurrence of a HPV related genetic aberration, the cell exceeding 9c would carry the aberration through the endoreduplicative cycles. The small fraction of the 9c exceeding cells, therefore, like the tip of an iceberg, reflects general genetic instability and early clonal changes in HSIL.

In three (23.1%) out of the 13 HSIL samples analyzed by FISH recurrent aberrant chromosome ratios could be observed. This was far more than it could be explained by random mechanisms. The same type os imbalance could further be followed in 5c-9c gated cells in all of the three mentioned samples in a significantly lower degree. The imbalance was, however, not evident when the usual amount of

200 cells was randomly evaluated following FISH. The recurrent FISH signal imbalance is a strong argument for a common origin of the involved cells. This feature suggests that highly aneuploid cells of HSIL accumulate clonal genetic aberrations that are not detectable in peridiploid squamous cells in early progressive lesions. In the follow-up of our analyzed cases patients no. 9 and 13 developed cervical intraepithelial neoplasia (CIN I and III), while no histological report could be achieved from the third patient (no. 6, *Table 1*.)

In summary, polyploid cells in HSIL present with significant information on HPV-induced genetic instability. These cells can be easily selected on the basis of their highly increased nuclear DNA content from routine cytological samples by automated procedures. Further to numerical chromosomal abnormalities, the occurrence of which was demonstrated in this work, more specific genetic aberrations are to be expected. A detailed analysis of events exceeding 9c also promise a predictive potential for the monitoring of patients with persistant HPV infection.

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