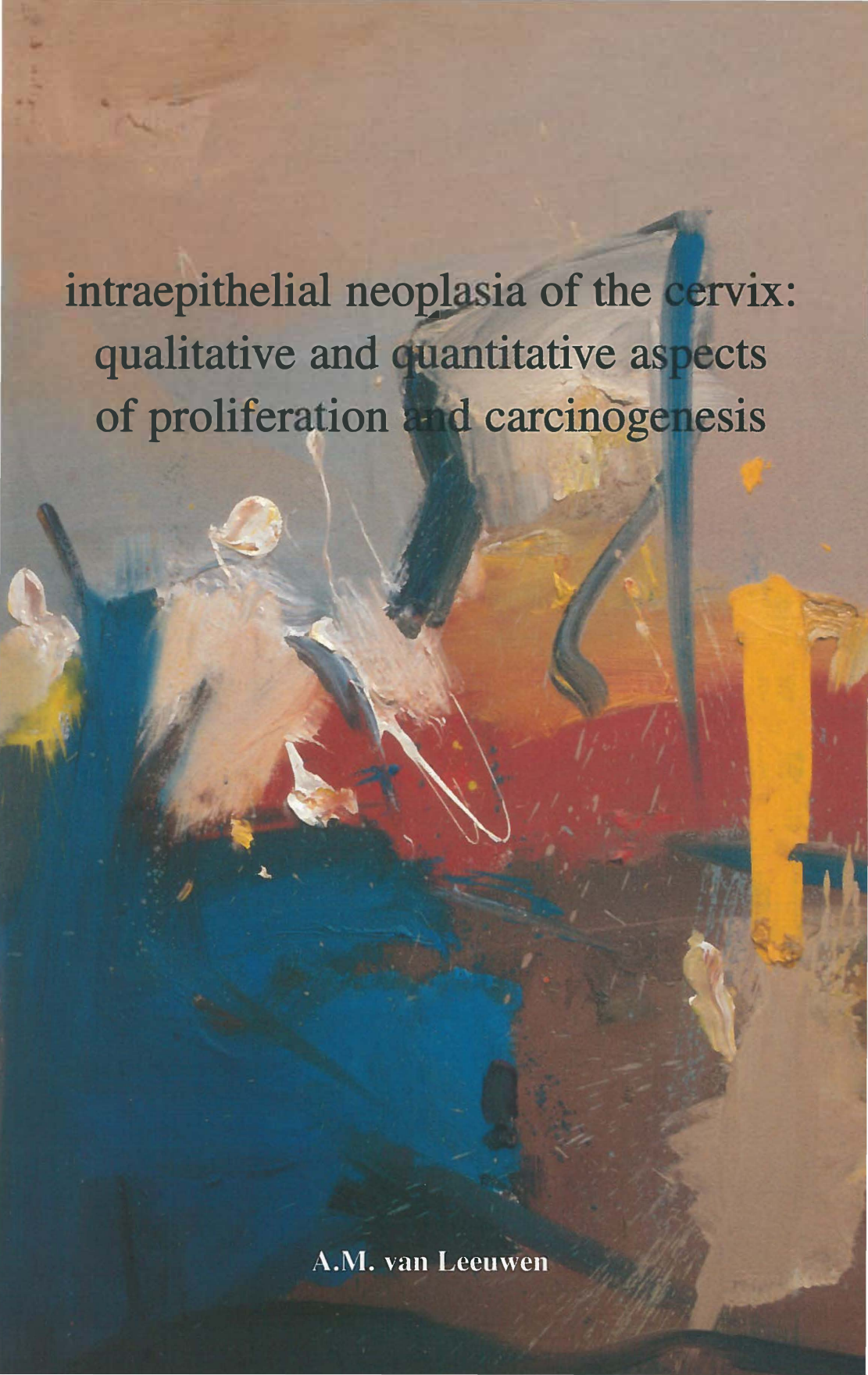
intraepithelial neoplasia of the cervix: qualitative and quantitative aspects of proliferation and carcinogenesis



A.M. van Leeuwen

intraepithelial neoplasia of the cervix: qualitative and quantitative aspects of proliferation and carcinogenesis

A.M. van Leeuwen

Stellingen

behorende bij het proefschrift van

A.M. van Leeuwen

Intraepithelial neoplasia of the cervix: qualitative and quantitative aspects of proliferation and carcinogenesis.

Vergeleken met de conventionele CIN classificatie leidt het classificeren en tellen van mitosen in premaligne cervix-afwijkingen niet tot een betere overeenkomst tussen de biopsie diagnose enerzijds en de definitieve diagnose anderzijds.

Mutaties in het p53-gen spelen een ondergeschikte rol in de pathogenese van het plaveiselcelcarcinoom van de cervix uteri.

Beeldvormende technieken als DNA-cytometrie zijn waardevol in kankeronderzoek, echter op zichzelf niet bruikbaar als diagnosticum bij patiënten met een afwijkende cervix uitstrijk.

De ontstaanswijze van lag-type mitosen is nog niet opgehelderd.

Bij het bepalen van het humane papillomavirus in routine cervix uitstrijkjes van gezonde vrouwen dienen kosten en effectiviteit in ogenschouw te worden genomen.

Conservatief beleid bij een zwangere patient met CIN III is verantwoord onder strikte cytologische en coloposcopische evaluatie.
Guintoli R et al, Gynecol Oncol 1991;42:68-73.

Bacteriele vaginose is niet gerelateerd aan het ontstaan van plaveiselcelcarcinoom van de cervix uteri.
Peters N et al, Sex Trans Dis 1995;22:296-302.

Het bepalen van de zygotie van meerlingen op basis van de morfologie van de lagen van het tussenschot van de placenta kan vervangen worden door microsateliet analyse van DNA uit de navelstrengen.

Het is een kwestie van "pech" dat uit die miljoenen cellen juist die ene cel ontaardt tot een maligne cel.
R Dole, NRC handelsblad november 1997.

Indien de kosten voor kinderopvang integraal aftrekbaar zijn van de belasting zullen meer vrouwen blijven werken.

De invoering van een co-assistentschap pathologie kan leiden tot een generatie klinici die de ogen open houdt tijdens de donkere momenten in de klinisch-pathologische besprekingen.

Specialiseren, promoveren en baren voor je vijfendertigste mag niet leiden tot saaiheid tot je vijfenzestigste.

Coverpage: Oilpainting without title, painted by H. van Tongeren, 1977
Reproduced with kind permission of the painter

Wie niet waagt, wie niet wint.

Aan papa en mama.

RIJKSUNIVERSITEIT GRONINGEN

INTRAEPITHELIAL NEOPLASIA OF THE CERVIX: QUALITATIVE AND QUANTITATIVE ASPECTS OF PROLIFERATION AND CARCINOGENESIS

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de Rector Magnificus, Dr. F. van der Woude
in het openbaar te verdedigen op
woensdag 4 februari 1998
des namiddags te 4.15 uur

door

Antonia Maria van Leeuwen
geboren op 14 juli 1964,
te Hazerswoude

Promotores : Prof. Dr. J.G. Aalders
Prof. Dr. J.D. Elema
Prof. Dr. M.P.M. Burger

Referent : Dr. H. Hollema

ISBN-nummer: 90 - 90 11312-6

Contents

CHAPTER 1

INTRODUCTION

Introduction

Cervical cancer is the fourth most common cancer among women worldwide preceded by breastcancer, lungcancer and cancer of colon and rectum[13]. Annually there are approximately 465,000 new cases and more than 200,000 deaths. In the third world cervical cancer is the most common cancer. This stands in contrast to the United States and Europe where incidences of cervical cancer have been declining[19]. In the Netherlands 12.5 new cases per 100,000 people a year are diagnosed and annually 300 women die from cervical cancer. Screening, by cytological examination of exfoliating cervical cells, is a good indicator of invasive and pre-invasive cancer[67] and has influenced the mortality rate.

Histology and classification

The cervix uteri is located in such a way that examination is relatively simple. Due to this location the cervix uteri is extensively studied, which has resulted in some understanding of the pathogenesis of squamous cervical cancer.

Squamous cervical cancer arises from precursor lesions. These precursor lesions usually develop in the transformation zone, the place where columnar and squamous epithelium meet. During adolescence the squamocolumnar junction is usually located on the ectocervix. Because of environmental factors in the vagina the fragile columnar epithelium responds by undergoing a change from basal cell hyperplasia to immature squamous metaplasia and, finally, mature squamous metaplasia. It is in this area where most precursor and invasive squamous carcinomas develop[1,4]. There are several classification systems for grading precursor lesions, which are nicely described by Pieters[75]. In 1968, Richart[83] introduced the definition of cervical intraepithelial neoplasia (CIN); graded into CIN I, CIN II, and CIN III, histologically parallelled by increased nuclear atypia, increased disturbance of cellular differentiation, epithelial architecture and increased mitotic activity. This classification has been adopted by the world health organization and has found general acceptance[80].

The CIN classification is hampered by two major problems. In the first place many follow up studies have shown that CIN lesions harbour an increasing risk of developing

carcinoma with increasing CIN grade. But the chance of developing invasive carcinoma in an individual patient cannot be estimated. This is partly due to the differences in the results of follow-up studies and to the differences in interpretation of cytological and histological features and to different statistical approaches of epidemiological data[7,32,40,41,47,49,50,53,55,56,60,64,65,71,73,82,83,110]. Nasiell et al followed, cytologically, 894 women with CIN II. Patients entered the study after one smear indicating moderate dyskaryosis. In 54% of the patients biopsies were taken showing regression in 50%, persistence in 15% and progression in 35%. Three patients (0.3%) developed invasive carcinoma[64]. Fox et al studied 278 patients with mild or moderate dyskaryotic smears. Persistence occured in 9%, regression in 31% and progression to CIN III or invasive carcinoma in 59% and 1% respectively[33]. Green and Donovan found progression to invasive carcinoma in patients with CIN III in exconization specimen of 0.17% and stated that progression from CIN III to invasive carcinoma takes 20 years[40]. A group of 948 patients with CIN III in biopsy or exconization specimens were followed for 5 to 28 years. Out of these 948 cases, 131 showed persistent abnormal pap smears, 29(22%) of whom developed invasive carcinoma.

It is difficult to compare these studies. Some showed low progression rates, probably due to the fact that the lesions were removed for diagnosis during the study which is also stated by Struyk who demonstrated that by taking biopsies the course of the CIN lesion is changed[101]. On the other hand cytological follow up alone could miss a micro-invasive carcinoma. Despite all the critiscism on these studies, it has become clear that CIN III lesions bear a substantial risk of developing invasive carcinoma and should therefore be treated[31,71] by radical excision.

CIN I and CIN II lesions have a low risk of developing invasive carcinoma, but some cases of cervical carcinoma, apparently arising from CIN I or CIN II, have been described[32,55,84].

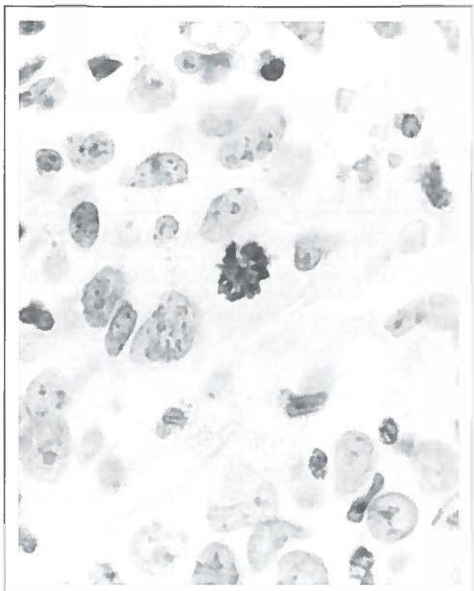
The second problem is the high inter- and intraobserver variation among pathologists in grading CIN. Ismail et al assessed the variability of grading 100 cervical biopsies among 8 experienced histopathologists. They concluded that the agreement was excellent for invasive lesions, moderate for CIN III, and poor for CIN I and CIN II. Especially the distinction between reactive squamous epithelial lesions and CIN I was an important source of disagreement[46]. Similar conclusions were drawn from a study by

Robertson et al, in which the interobserver agreement between 10 pathologists in grading 100 cervical specimens was studied[86]. In a multicenter study in the northern part of the Netherlands interobserver agreement among 10 pathologists was studied. One hundred slides containing normal epithelium (no dysplasia), mild dysplasia, moderate dysplasia, severe dysplasia, and carcinoma in situ were examined by individual pathologists. These results were compared with a concensus diagnosis made by three experienced pathologists. Mismatches were weighted on the severity of mismatch by multipling by "1" or "2". The individually weighed kappa scores ranged from 0.65 to 0.26 with best agreement for carcinoma in situ. The worst agreement was found for mild dysplasia[76].

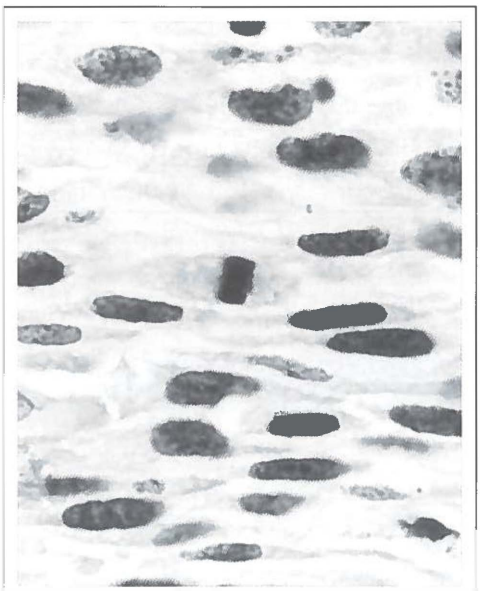
Several attempts have been made to solve this problem by the development of new classification systems. One of these is a proposal for a binary classification system; the Bethesda system[37,66] in which CIN I and koilocytotic lesions represent low grade squamous intraepithelial lesions (low grade SIL) and CIN II and CIN III high grade SIL; this system has found many adherents in the United States. Agreement studies with the Bethesda system report good agreement among pathologists[37]. This is to be expected because reducing the number of choices will increase the likelihood of agreement based on chance alone. Genest et al studied the agreement among 4 pathologists in the classification of 75 cervical biopsies with squamous lesions (Bethesda system). Three grades were available; low, indetermined and high. The consistency within the indetermined group varied around 0. Within the high SIL group there was good to excellent agreement (kappa scores ranging from 0.43 to 0.63). The kappa values within the low SIL group ranged from 0.36 to 0.61[37].

Morris suggested a classification based on a 100 point scale to minimize interobserver disagreement[62]. Anderson et al described the interpretation and criteria for the CIN grades in an attempt to achieve more consensus among pathologists[2]. They stressed the importance of the presence or absence of atypical mitotic figures.

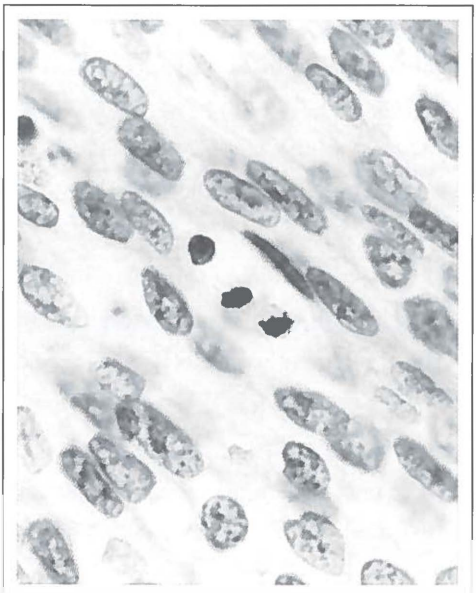
Which of the CIN lesions progresses to invasive cancer in an individual patient cannot be predicted merely on the basis of histomorphology alone. Numerous studies of CIN related parameters were caried out. Among these, proliferation rates and atypical mitotic figures, human papillomavirus and abnormal cellular DNA contents came up as progression related features, which will be described in more detail further on.



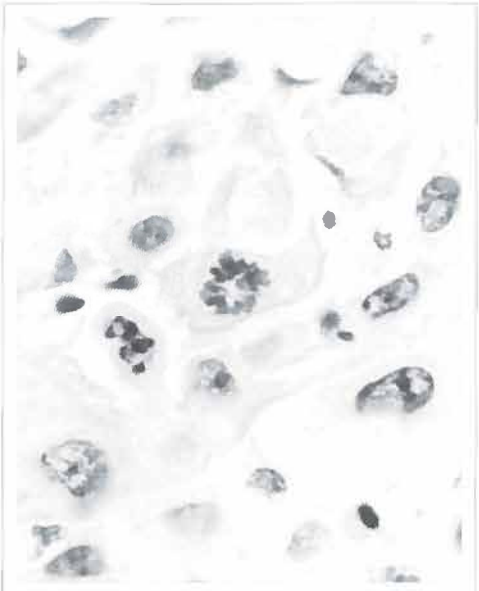
Normal mitosis:
prophase



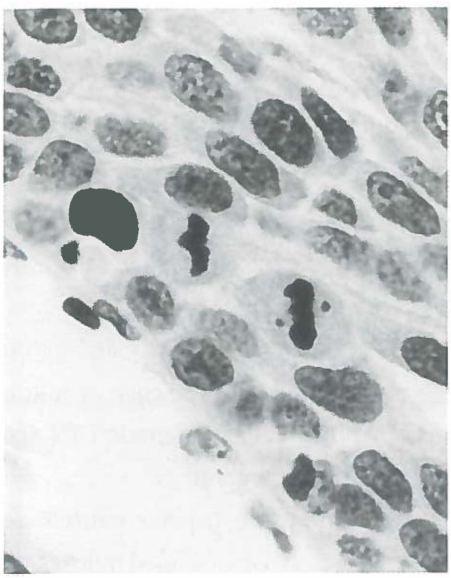
Normal mitosis:
metaphase



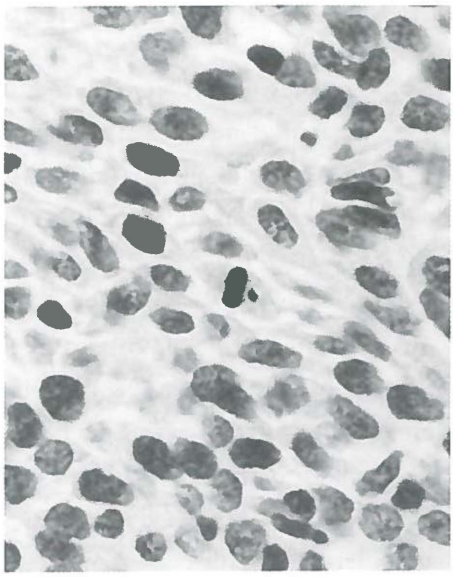
Normal mitosis:
anaphase



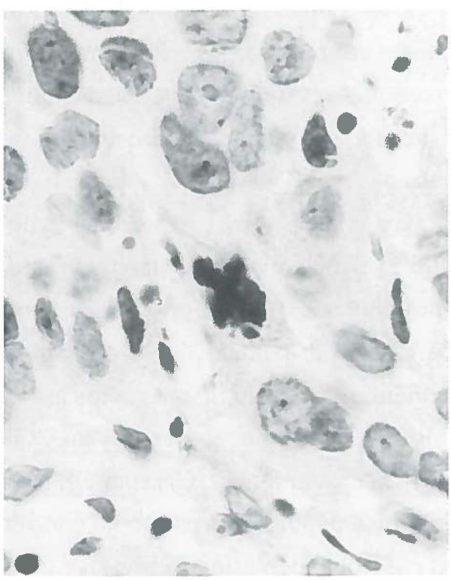
Abnormal mitotic figure:
ringmitosis



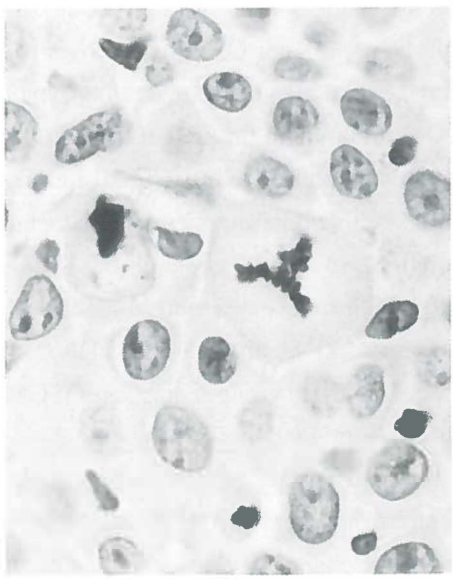
Abnormal mitotic figure:
three group metaphase



Abnormal mitotic figure:
two group metaphase



Abnormal mitotic figure:
quadripolar mitosis



Abnormal mitotic figure:
tripolar mitosis

Mitotic activity and atypical mitotic figures.

Disregulation of the cell-cycle resulting in an increased proliferation rate and abnormal mitotic figures are the characteristics of many malignancies. Quantification of mitotic activity is one of the major criteria for the diagnosis of malignant soft tissue tumors. The importance of mitotic activity is further illustrated by its prognostic significance in breast carcinomas[5,24] and studies on advanced stage ovarian carcinoma, where the mitotic activity index correlated well with tumor grade[16].

In contrast with normal cervical epithelium in CIN the mitotic activity and mitotic figures are not restricted to the basal layer of the epithelium. The presence of mitotic figures in the higher levels of the epithelium is an argument for high grade CIN. The actual quantification of mitotic activity has been given little attention[22,102].

Eyecatching atypical mitotic figures are multipolar mitoses; tripolar mitoses and quadripolar mitoses, dispersed mitotic figures, ring mitoses, V or C-shaped mitoses and the so-called lag type mitoses. Multipolar mitoses are metaphases with an abnormal configuration of the equatorial plate; the chromosomes are located along several radial axes. The tripolar mitoses and the quadripolar mitoses are the most common. Dispersed mitoses are giant or balloon mitoses, in which the chromosomes lie dispersed within the mitotic area. V- or C shaped mitoses or ring mitoses are metaphases with an abnormal shape of the metaphase plate. Lag type mitoses are mitoses with non-attached condensed chromatine in the area of the mitotic figure. The three group metaphase and the two group metaphase are the two most easily recognized.

Dustin and Parmentier demonstrated that lag type mitoses can be induced by hydroquinone, which arrests the mitotic sequela[30,69,70]. In induced cervical carcinoma in mice, lagging chromosomes were considered to be a morphological indicator of an abnormal amount of DNA and abnormal DNA karyotypes[93]. Also in humans, lag type mitoses, especially three group metaphases (3GM), were more often found in aneuploid cervical lesions compared to polyploid and diploid lesions[51,52,76,77,113,119]. In a recent study the 3GM was found in 92% of CIN lesions adjacent to invasive carcinoma[63]. Claas et al (1992) demonstrated that the 3GM and the presence of human papillomavirus can be considered to be characteristics indicating an increased risk for progression to invasive carcinoma[23]. Moreover, atypical mitotic figures and a high mitotic index were also indicative of dysplasias of the gastro-intestinal tract[91,92].

Quantification and qualification of mitoses in cervical lesions is never incorporated into daily practice, partly due to 1) interobserver disagreement[28,99,120] but probably also because most pathologists believe that 2) mitoses counting is time-consuming and boring[6,45,97] and 3) that mitotic activity is influenced by fixation. The reproducibility of mitoses counting among 9 pathologists was studied in 10 lymphomas. The agreement among the 4 most experienced pathologists was good; they counted comparable mean numbers of mitoses per square mm[28]. Good interobserver agreement was found in a recent study by van Diest et al; they performed a multicentre study in which mitotic figures were counted according to a detailed protocol[104]. In the latter study mitoses were counted in a reasonable timespan (about 10 minutes)[104]. Concerning the boredom of mitoses counting, this is a more personal discussion. The third argument is discussed in a study by Donhuijsen et al[29]. They demonstrated that the recognition of mitotic figures was influenced by delayed fixation, whereas the total number of mitoses was not altered[29].

Human papillomavirus.

The significance of human papillomavirus for the genesis of cervical carcinoma has been the subject of many studies[96], and it has become clear that HPV is strongly correlated with the development of cervical squamous carcinoma[17,56,95]. The reported prevalence of HPV in cervical carcinoma varied from 83% to 100%, with HPV 16 and 18 as the dominant types[14,34,58,87,112]. Lymphnode metastases contain the same HPV type as the primary carcinoma, which also stresses the intimate relation between HPV and cervical carcinoma[57]. Compared to carcinoma, CIN lesions exhibit a more divers pattern of HPV types. The types 6 and 11 are more commonly found in the lower CIN grades, whereas the oncogenic types 16, 18, 31, and 33 are more often found in higher CIN grades[96]. In a recent study 265 consecutively collected patients with persistent mild to moderate dyskaryosis or severe dyskaryosis were analysed for the presence of HPV detection by PCR. No HPV was found in 60(22%) cases, 108(41%) showed type 16, 19(7%) type 18, 27(10%) showed type 31 or 33 and the remaining 20% showed more than one HPV type. Out of 180 CIN III cases 74% demonstrated HPV 16, 18, 31, 33 or a combination of these types[18], which further supports the connection between HPV and the development of cervical cancer. Recent studies have also shown that women with a normal cervical

smear, but with HPV type 16, are more likely to proceed to squamous intraepithelial lesions[43,54] or to have histological proven CIN[38,43,95]. Currently more than 70 different HPV types are identified. A new type is defined when less than 90% homology is found in the early genes E6 and E7 and the late gene L1 regions, compared with other known HPV types[27]. Van Ranst et al constructed a phylogenetic tree on the basis of the alignment of the E6 sequence of 28 HPV types and demonstrated that types 16, 31 and 33, which are commonly found in cervical cancer are located on the same main branch[109].

The viral proteins of the early (E) genes E6 and E7 are involved in transactivation of the viral promotor and, in case of the oncogenic types, in transformation of the host cell. Both proteins interfere with cell cycle control[26]. E6 binds the transcription regulatory protein p53, resulting in the ubiquitin-dependent degradation of p53[94]. Wild type p53 arrests the cell-cycle in respons to DNA damage and is therefore a tumorsupressor gene. Loss of p53 function causes accumulation of mutations in successive generations of cells. E7 binds to retinoblastoma tumorsupressorgene product (pRB)[31]. pRb is a nuclear protein and is activated by dephosphorylation. Activated pRB results in the release of nuclear factors essential for cell proliferation. E7 may also stimulate cells to proceed through G2 to cell division by an interaction of E7 with cycline and the cycline-dependent kinase cdk2[103]. This interaction disturbs the normal regulation of entering into mitoses. Moreover E7 interferes with the transcriptional suppression of c-myc, and HPV integration occurs often near the c-myc gene[25]. Elevated c-myc levels stimulate keratinocyts to proliferate[75]. In collaboration with activated RAS, E7 is able to transform foetal rat kidney cells[74].

DNA analysis

As early as 1902 Boveri suggested that malignancy might result from an unbalanced set of chromosomes[15]. Numerical as well as structural chromosomal changes occur. Numerical changes indicate the gain or loss of whole chromosomes, whereas structural changes occur in rearrangement of chromatine, as in translocation. Changes in the amount of DNA can quantitatively be examined by flow cytometry or image-cytometry. Qualitative analysis can be done by karyotyping, in situ hybridisation or PCR analysis. The techniques for qualitative analysis will not be discussed further, because it is beyond the scope of this thesis.

Flow cytometry allows DNA ploidy analysis of a large number of nuclei in a short time, but it is not possible to study individual nuclei in detail. Image cytometric analysis also allows DNA ploidy analysis, but provides information on individual nuclei, too.

The flow or image cytometric DNA ploidy patterns can be visualized by histograms displaying the amount of DNA present in stemlines[118]. The histograms may be assessed as DNA-diploid, DNA-polyploid and DNA-aneuploid, according to the following definitions[3,44,116]. In a diploid histogram a distinct G0/G1 peak (corresponding to cells in the rest phase of the cell cycle) is found in the diploid (2C±0.1C) region with a small portion of cells in the S phase (synthese) and in the G2/M phase (corresponding to the G2 and mitotic phase). This pattern is usually seen in normal tissue. A polyploid histogram shows distinct peaks in the tetraploid (4C±0.2C) or octaploid (8C±0.4C) regions. All other patterns are aneuploid histograms.

The visual classification of DNA histograms into diploid, polyploid or aneuploid is often subjective[116]. An alternative approach is to use ploidy related descriptors, such as the 5C-exceeding rate (5CER) and the 2C deviation index (2CDI)[11,12]. The 5CER is the proportion of cells in the analysed population with a DNA content of more than 5C. The 2CDI is the mean square deviation between the DNA content in the selected cells and the diploid DNA content.

Flow cytometrical analysis on cervical carcinomas has been done in various studies[3,48,61,100] without evidence that ploidy predicts prognosis, as does lymphenode involvement[48,100]. Out of the preinvasive lesions, diploid or polyploid lesions are more likely to regress or persist, whereas aneuploid lesions often progress[10,35,59]. Moreover, morphological features related to high grade CIN, such as the presence of the three group metaphase[77] or infection of human papillomavirus, are more likely to be aneuploid[9,36,83,98,119]. The next step was to perform DNA cytometry on cervical scrapes. The results of the first studies were very promising [7,12,21,39,42,67,87,111,114,115,117]. This has resulted in attempts to automate the screening process and various automated systems have been developed[8]. In short, most systems use monolayers of cells of which density, diameter and shape are measured and compared to a standard population. Unfortunately, due to various reasons none of the machines was found acceptable for cytological screening in a daily routine setting.

One of the automated systems, the LEYTAS (Leyden Television Analysis System) performs automated cell selection based on nuclear density and size, and measures geo-

metrical and densitometrical parameters[107]. Standard routines of equipment include artefact rejection tests and selected objects can be projected on a TV screen[78,79]. This facilitates a final visual step to remove any remaining artefacts or overlapping cells from the data set. In a study performed with the use of this machine, the number of cells exceeding 5C strongly correlated with precancerous or cancerous lesions of the cervix[80]. In another study 1500 cervical smears were examined by this machine[79]. Out of the 321 cases with severe dysplasia, 320 (99.7%) were selected by the machine, and out of the 561 negative smears, 90 (16%) were classified as dysplasia. The great advantage of this system is the automated cell selection, because interobserver variation has been reported[20], and the visualization of the selected items on a TV screen. Disadvantages are the slide preparation which is still a time-consuming and laborious task[103-105], and the flow-speed of the machine: 10-15 minutes per slide.

In summary, it has become clear that high grade CIN lesions bear substantional risk of developing invasive carcinoma. These high grade CIN lesions often harbour oncogenic HPV types, are more often aneuploid and show high mitotic indices and atypical mitotic figures. Taking this knowledge into account, the following studies were performed.

General outline

The aim of this study was threefold.

In the first place, in order to remove the above mentioned problems concerning the CIN classification we studied whether an objective and easily recognizable morphological parameter could contribute to the definition of a subpopulation of CIN lesions, harbouring a high risk of developing invasive carcinoma. From previous studies atypical mitotic figures, especially the threegroup metaphase (3GM), came up as a possible candidate. To answer this question, mitotic figures were analysed and the mitotic index was assessed in, on the one hand, a large series of prospectively collected women with cervical intraepithelial neoplasia (chapter 2) and, on the other hand, in a group of women with either microinvasive carcinomas or non-neoplastic cervical intraepithelial neoplasia (chapter 3). This has resulted in a pathogenetic model, which was applied on a series of women with abnormal cervical cytology, whose complete transformation zone was available for histological examination (chapter 4).

In the second part, we studied the relation between lag type mitoses and human papillomavirus and p53 expression. This was done in order to understand the biological context in which these mitoses occur. Chapter 5 describes a series of CIN III lesions in which lag type mitoses and the mitotic index were related to the human papillomavirus type. Human papillomavirus interacts with cell cycle mediators such as p53. P53 plays an important role in the development of the nuclear spindle. Spindle malformation may result in atypical mitotic figures. In chapter 6 we examined the expression of p53 in relation to mitotic index and lag type mitoses in CIN II and III lesions.

In the third place, we studied aneuploid cervical smears, since these lesions progressed more often to higher grades than euploid or polyploid lesions. In chapter 7 the number of cells exceeding 5C as a descriptor of aneuploidy, and human papillomavirus were studied in dyskaryotic cervical smears, in order to get insight in the role of HPV infections in the development of cells with a DNA content exceeding 5C. Chapter 8 demonstrates the utility of aneuploidy related parameters (number of cells exceeding 5C, number of non-polyploid cells exceeding 5C, 5C exceeding rate and 2C deviation index) as a screening parameter in a large group of consecutively collected women with abnormal cervical smears.

Finally, the chapters are summarized and discussed (chapter 9).

References

1. Abdul-Karim FW, Fu YS, Reagan JW, Wentz WB. Morphomeric study of intraepithelial neoplasia of the uterine cervix. Obstet Gynecol 1982;60:210-4.
2. Anderson MC, Brown CL, Buckley CH, Fox H, Jenkins DG, Manners BTB, Melchers DH, Robertson AJ, Wells M. Current views on cervical intraepithelial neoplasia. J Clin Pathol 1991;44:969-78.
3. Atkin NB, Kay R. Prognostic significance of modal DNA value and other factors in malignant tumors, based on 1465 cases. Br J Cancer 1979;40:210-21.
4. Autier P, Coibion M, Huet F, Grivegnee AR. Transformation zone location and intraepithelial neoplasia of the cervix uteri. Br J Cancer 1996;74:488-90.
5. Baak JPA, van Dop H, Kurver PHJ et al. The value of morphometry to classic prognosticators in breast carcinoma. Cancer 1985;56:374-82.
6. Baak JPA. Mitosis counting in tumors. Hum Pathol 1990;21:683-4.
7. Bacus JW, Wiley EL, Galbraith W, Marshall PN, Wilbanks GD, Weinstein RS. Malignant cell detection and cervical cancer screening. Analyt Quant Cytol Histol 1984;6:121-130.
8. Banda-Gamboa H, Ricketts I, Cairns A, Hussein K, Tucker JH, Husain N. Automation in cervical cytology: an overview. Anal Cell Pathol 1992;4:25-48.
9. Bergeron C, Barasso R, Beaudenon S, Flamant P, Croissant O, Orth G. Human papilloma viruses associated with cervical intraepithelial neoplasia. Great diversity and distinct distribution in low-and high-grade lesions. Am J Surg Path 1992;16:641-9
10. Bibbo M, Dytch HE, Alenghat E, Bartels P, Wied GL. DNA ploidy profiles as prognostic indicators in CIN lesions. AM J Clin Pathol 1989;92:261-5.
11. Böcking A, Adler CP, Common HH, Hilgarth M, Grantzen B, Auffermann W. Algorithm for a DNA-cytophotometric diagnosis and grading of malignancy. Anal Quant Cytol 1984;6:1-6.
12. Böcking A, Higarth M, Aufferman W, Hack-Werdier C, Fisher-Becker D, von Kalkreuth G. DNA-cytometric diagnosis of prospective malignancy in borderline lesions of the uterine cervix. Acta Cytol 1986;30:607-15.
13. Boring CC, Squires TS, Tong T, Montgomery S. Cancer statistics, 1994. CA 44:7, 1994.
14. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 1995;87:765-802.
15. Boveri T. Uber mehrpolige mitosen als mittel zur analyse des zellkerns. Verh Phys Med Ges., Würtzburg, Germany, 1902.
16. Brinkhuis M, Baak JPA, Meijer PJ, van Diest PJ, Mogensen O, Bichel P, Neijt JP. Value of quantitative pathological variables as prognostic factors in advanced ovarian carcinoma. J Clin Pathol 1996;49:142-8.
17. Brinton LA. Epidemiology of cervcical cancer-overview. In: The epidemiology of cervical cancer and human papillomavirus. N. Munoz, Bosch FX, Shah KV, Meheus A (eds). Lyon, International agency for research on cancer 1992 p3-23.
18. Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predictive value of human papillomavirus type for the histological diagnosis of women with cervical cytological abnormalities. Br Med J 1995;310:94-5.
19. Cancer fact and figs. 1993, American cancer society, p13.
20. Carey FA, Salto-Tellez M, Kelly C, Dye R, Duvall E, Lamb D. Interobserver variation in cell selection for DNA image cytometry. J Clin Pathol 1995;48:616-9.
21. Chatelain R, Schnuck T, Schindler EM, Schindler AE, Böcking A. Diagnosis of prospective malignancy in koilocytotic dysplasias of the cervix with DNA cytometry. J Reprod Med 1989;34:505-10.

22. Chi CH, Rubio CA, Lagerlöf. The frequency and distribution of mitotic figures in dysplasia and carcinoma in situ. Cancer 39;1977:1218-23.
23. Claas ECJ, Quint WGV, Pieters WJLM, Burger MPM, Oosterhuis WJW, Lindeman J. Human papillomavirus and the three group metaphase figure as a markers for an increased risk for the development of cervical carcinoma. Am J Pathol 1992;140:497-502.
24. Clayton F. Pathologic correlates of survival in 378 lymph node negative infiltrating ductal carcinomas: Mitotic count is the best single predictor. Cancer 1991;II:1309-17.
25. Couturier J, Sastre-Garau X, Schneider-Manoury S, Labib A, Orth G. Integration of human papillomavirus DNA near myc genes in genital carcinomas and its consequence for proto-oncogene expression. J Virol 1991;65:4534-4538.
26. Cordon-Cardo C. Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 1995;147:545-60.
27. De Villiers EM. Hybridisation methods other than PCR: an update. In: The epidemiology of human papillomavirus and cervical cancer, edited by Munoz N, Bosch FX, Shah KV and Meheus A. Oxford, UK: Oxford University Press, p. 111-113.
28. Donhuijsen K. Mitosis counts: Reproducibility and significance in grading malignancy. Hum Pathol 1986;17:1122-5.
29. Donhuijsen K, Schmidt U, Hirche H, van Beuningen D, Budach V. Changes in mitotic rate and cell cycle fractions caused by delayed fixation. Hum Pathol 1990;21:709-14.
30. Dustin P, Parmentier R. Donnees experimentales sur la nature des mitoses anormales observees dans certains epitheliomas du colin uterin. Gynecologie et Obsterique 1953;52:258-65.
31. Dyson N, Howley PM, Münger K, Harlow E. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989;243:934-40.
32. Ferenzy A, Winkler B. Cervical intraepithelial neoplasia and condyloma. In: Kurman RJ, ed. Blaustein's pathology of the female tract. 3rd ed. New York: Springer-Verlag 1987, 177-217.
33. Fox CH. Biologic behavior of dysplasia and carcinoma in situ. Am J Obstet Gynecol 1967;99:960-74.
34. Franco EL. Meeting report 13th international papillomavirus conference. Papillomavirus Rep. 1994;5:183-7.
35. Fu YS, reagan JW, richart RM. Definition of precusors. Gynecol Oncol 1981;12:220-31.
36. Fuji T, Crum CP, Winkler B, Fu YS, Richart RM. Human papillomavirus infection and cervical intraepithelial neoplasia: histopathology and DNA content. Obstet Gynecol 1984;63:99-104.
37. Genest DR, Stein L, Cibas E, Sheets E, Zitz JC, Crum CP. A Binary (Bethesda) sytem for classifying cervical cancer precursors. Hum Pathol 1993;24:730-6.
38. Goff BA, Muntz HG, Bell DA, Wertheim I, Rice LW, Human papillomavirus typing in patients with papanicolaou smears showing squamous atypia. Gynecol Oncol 1993;48:384-88.
39. Göppinger A, Freudenberg, Ross A, Hillemans HG, Hilgarth M. The prognostic significance of DNA distribution in squamous-cell carinomas of the uterine cervix. Anal Quant Cytol Histol 1986;2:148-51.
40. Green GH, Donovan JW. The natural history of cervical carcinoma in situ. J Obstet Gynecol Brit Commonwealth 1979;77:1-9.
41. Hall JE, Walton L. Dysplasia of the cervix: A prospective study of 206 cases. Am J Obstet Gynecol 1968;100:662-71.
42. Hanselaar AGJ, Vooijs GP, Mayall BH, Pahlplatz MMM, Van 't Hof-Grootenboer AE. DNA changes in progressive cervical intraepithelial neoplasia. Anal Cell Pathol 1992;4:315-24.

43. Herrington CS, Evans MF, Hallam NF, Charnock FM, Gray W, McGree JO'D. Human papillomavirus status in the prediction of high-grade cervical intraepithelial neoplasia in patient with persistent low-grade cervical cytological abnormalities. Br J Cancer 1995;71:206-9.
44. Hiddeman W, Schuman J, Andreef M et al. Convention on nomenclature for DNA cytometry. Cytometry 1984;5:445-6.
45. Hilsenbeck SG, Allred DG. Improved methods of estimating mitotic activity in solid tumors. Hum Pathol 1992;23:601-2.
46. Ismail SM, Colclough AB, Dinnen JS, Eakins D, Evans DMD, Gradwell E, O'Sullivan JP, Summerell JM, Newcombe RG. Observer variation in histopathological diagnosis and grading of cervical intraepithelial neoplasia. BMJ 1989;298:707-10.
47. Jones MH, Jenkins D, Cuzick J et al. Mild cervical dyskaryosis: safety of cytological surveillance. Lancet 1992;339:1440-3.
48. Kenter GG, Cornelisse CJ, Jiwa NM, Aartsen EJ, Hermans J, Mooi W, Heintz APM, Fleuren GJ. Human papillomavirus type 16 in tumor tissue of low-stage squamous carcinoma of the uterine cervix in relation to ploidy grade and prognosis. Cancer 1993;71:397-401.
49. Kinlen LJ, Springgs AI. Women with positive cervical smears without surgical intervention. A follow up study. Lancet 1978;2:463-5.
50. Kirkland JA. Mitotic and chromosomal abnormalities in carinoma in situ of the uterine cervix. Acta Cytol 1966;10:81-6.
51. Kirkland JA, Stanley MA, Cellier KM. Comparative study of histologic and chromosomal abnormalities in cervical neoplasia. Cancer 1967;20:1934-52.
52. Kiviat NB, Critchlow CW, Kurman RJ. Reassessment of the morphological continuum of cervical intraepithelial lesions: does it reflect different stages in the progression to cervical carcinoma. In: The epidemiology of cervical cancer and human papillomavirus. Munoz E, Bosch FX, Shah KV, Meheus A (eds). International Agency for Research on Cancer, Lyon. p59-66.
53. Kjaer SK, Van den Brule AJC, Bock JE, Poll PA, Engholm G, Sherman ME, Walboomers JMM, Meijer CJLM. Human papillomavirus. The most significant risk determinant of cervical intraepithelial neoplasia. Int J Cancer 1996;65:601-6.
54. Koss LG, Stewart FW, Foote FW, Jordan MJ, Bader GM, Day E. Some histological aspects of behaviour of epidermoid carcinoma in situ and related lesions of the uterine cervix. Cancer 1963;16:1160-1211.
55. Koutsky LA, Holmes KK, Critchlow CW, Stevens CE, Paavonen J, Beckman AM, DeRouen TA, Galloway DA, Vernon D, Kiviat NB. Cohort study of risk of cervical intraepithelial neoplasia grade 2 or 3 associated with cervical papillomavirus infection. New Engl J Med. 1992;327:1272-8.
56. Lancaster WD, Castello C, Santos C. Human papillomavirus deoxyribonucleic acid in cervical carcinoma from primary and metastatic sites. Am J Obstet Gynecol 1986;154:115-9.
57. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogential types. Obstet Gynecol 1992;79:328-37.
58. Mariuzzi G, Santinelli, Valli M, Sisti S, Montironi R, Marluzzi L, Alberti R, Pisani E. Cytometrical evidence that cervical intraepithelial neoplasia I and II are dysplasias rather than true neoplasias. An image analysis study of factors involved in the progression of cervical lesions. Anal Quant Cytol Histol 1992;14:137-47.
59. McIndoe WA, McLean MR, Jones RW, Mullins PR. The invasive potential of carcinoma in situ of the cervix. Obstet Gynecol 1984;64:451-8.

60. Merkel DE, McGuire WL. Ploidy, proliferative activity and prognosis. DNA flow cytometry of solid tumors. Cancer 1990;65:1194-1205.
61. Morris JA. Information and observer disargeement in histopathology. Histopathol 1994;25:123-8.
62. Mourits MJE, Pieters WJLM, Hollema H, Burger MPM. Three-group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 1992;167:591-5.
63. Nasiell K, Nasiell M, Vaclavinkova V. Behaviour of moderate cervical dysplasia during long-term follow-up. Obstet Gynecol 1983;61:609-14.
64. Nasiell K, Roger V, Nasiell M. Behaviour of mild cervical dysplasia during long-term follow-up. Obstet Gynecol 1986;67:665-9.
65. National Cancer Institute Workshop: The 1988 Bethesda system for reporting cervical/vaginal cytological diagnosis. JAMA 1988;262:931-4.
66. Nishiya I, Kibuchi T, Moriya S, Shimotomai K, Sawamura I. Acta Cytol 1977;21:271-5.
67. Papanicolaou GN, Traut HF: Diagnosis of uterine cancer by vaginal smears. New York the commonwealth fund, 1943.
68. Parmentier R, Dustin P. Early effects of hydroquinone on mitosis. Nature 1948;161:527-8.
69. Parmentier R, Dustin P. Reproduction experimentale d'une anomalie particuliere de la metaphase des cellules malignes (metaphase "A trois groupes"). Caryologia 1951;4:98-109.
70. Patten SF. Dysplasia of the uterine cervix. In: New concepts in gynecologic oncology, GC Lewis, WB Wentz RM Jaffe (eds). Philadelphia, Davis 33-44, 1966.
71. Paul C. The New Zealand cervical study: could it happen again? Br J Obstet Gynecol 1988;297:533-8.
72. Petersen O. Spontaneous course of cervical precancerous conditions. Am J Obstet Gynecol 1956;72:1063-71.
73. Phelps WC, Yee CL, Münger K, Howley PM. The human papillomavirs type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1a. Cell 1988;53:539-47.
74. Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Münger K, Howley PM, Moses HL. TGF-bl inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domeins. Cell 1990;61:777-85.
75. Pieters WJLM. De atypische mitose als kenmerk bij het klassificeren van plaveiselcelafwijkingen van de cervix uteri. Een evaluatie van morfologische criteria. Thesis in Dutch 1987.
76. Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW. The three-group metaphase is a morphological indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 1992;14:227-32.
77. Ploem JS, van Driel-Kulker AMJ, Goyarts-Veldstra L, Ploem-Zaaijer JJ, Verword NP, van der Zwan M. Image analysis combined with quantitative cytochemistry. Results and instrumental developments for cancer diagnosis. Histochem 1986;84:549-555.
78. Ploem JS, van Driel-Kulker AMJ, Ploem-Zaaijer JJ. Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. Path Res Pract 1989;185:671-5.
79. Ploem-Zaaijer JJ, Beyer-Boon ME, Leyte-Veldstra L, Ploem JS. Cytofluormetric and cytophotometric DNA measurements of cervical smears using a nex bi-color method. In: Tutorials of cytology , Chicago. Pressman NJ, Wied GL (eds). pp 225-35.
80. Poulsen HE, Taylor CW, Sobin LH. Histological typing of femal genital tract tumors. World Health Organization, Geneva 1975.
81. Prendville W, Guillebaud J, Bamford P, Beilby J, Steele SJ. Carcinoma of the cervix with recent normal papanicolaou tests. Lancet 1980;2:835-54.

82. Reid R, Crum CP, Herschman BR, Fu YS, Braun L, Shah KV, Agronow SJ, Stanhope R. Genital warts and cervical cancer. Subclinical papillomavirus infection and cervical neoplasia are linked by a spectrum of continous morphologic and biologic change. Cancer 1984;53:943-53.
83. Richart RM. Natural history of cervical intraepithelial neoplasia. Clin Obstet Gynecol 1968;10:748.
84. Richart RM. Causes and managment of cervical intraepithelial neoplasia. Cancer 1987:1951-7.
85. Richart RM, Barron BA. A Follow-up study of patients with cervical dysplasia. Am J Obstet Gynec 1969;195:386-93.
86. Riou G, Favre M, Jeannel D, Bourhis J, De Doussal V, Orth G. Association between poor prognosis in early-stage invasive cervical carcinomas and non-detection of HPV-DNA. Lancet 1990;335:1171-4.
87. Robertson AJ, Anderson JM, Swanson beck J, Burnett RA, Howatson SR, Lee FD, McLaren KM, Moss SM, Simpson JG, Smith GD, Tavadia HB, Walker F. Observer variability in histopathological reporting of cervical biopsy specimens. J Clin Pathol 1989;42:231-8.
88. Rosenthal DL, Suffin SC, Misserlain N, McLatchie C, Castleman KR. Cytomorphometric differences among individual "moderate dysplasia" cells derived from cervical intraepithelial neoplasia. Anal Quant Cytol 1984;6:189-95.
89. Rubio CA, Llatjos M. Kinetics of cell replication of the uterine cervix. IV. Proliferative loci in the basal layer. Acta Cytol 1982;26:367-70.
90. Rubio CA, Kirota T, Itabashi T. Atypical mitoses in elevated dysplasias of the stomac. Path Res Pract 1985;180:372-6.
91. Rubio CA. Atypical mitoses in colorectal adenomas. Path Res Pract 1991;187:508-13.
92. Scarpelli DG, von Haam E. A Study of mitoses in cervical epithelium during experimental inflamation and carcinogenesis. Cancer Res 1957;17:880-5.
93. Scheffner M, Wernesse BA, Huibregtse JM, levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990;1129-36.
94. Schiffman MH, Bauer HM, Hoover RN, Glass AG, Cadell DM, Rush BB, Scott DR, Sherman ME, Kurman RJ, Wacholder S, Stanton CK, Maos MM. Epidemiological evidence showing that human papilloma virus infection causes most cervical intraepithelial neoplasia. J Nat Cancer Inst 1993;85:958-64.
95. Schneider A, Koutsky LA. Natural history of epidemiological features of genital HPV infection. In: the epidemiology of cervical cancer and human papillomavirus. Munoz N, Bosch FX, Shah KV, Meheus A (eds). International agency for research on cancer, Lyon. 1992 IARC.
96. Scully RE. Mitosis counting-I and II. Hum Pathol 1992;7:481-4.
97. Shevchuk MM, Richart RM. DNA content of condyloma accuminata. Cancer 1982;49:489-92.
98. Silverberg SG. Reproducibility of the mitosis count in the histologic diagnosis of smooth muscle tumors of the uterus. Hum Pathol 1976;7:451-4.
99. Strang P, Stendahl U, Bergström R, Frankendal B, Tribukait B. Prognostic flow cytometric information in cervical squamous cell carcinoma: a multivariate analysis of 307 patients. Gynecol Oncol 1991;43:3-8.
100. Struyk APHB. Colposcopische evaluatie by afwijkende uitstrijk. Thesis in Dutch. Summary in english.
101. Tanaka T. Proliferative activity in dysplasia, carinoma in situ and microinvasive carcinoam of the uterine cervix. Path Res Pract 1986;181:531-9.
102. Tommassino M, Adamczewski JP, Carlotti F, Barth CF, Contorni M, Cavaliere F, Hunt T, Crawford L. HPV E7 protein associates with the proteinkinase p33-cdk-2 and cycline A. Oncogene 1993;8:195-202.
103. Van Diest PJ, Baak JP'A and 18 others. Reproducibility of mitosis counting in 2,469 breast cancer specimens. Results from the multicenter morphometric mammary carcinoma project. Hum Pathol 1992;23:603-7.

104. Van Driel-Kulker AMJ, Ploem-Zaaijer JJ, van der Zwan HJ, Tanke HJ. A preparation technique for exfoliated and aspirated cells allowing different staining procedures. Anal Quant Cytol 1980;2:243-6.
105. Van Driel-Kulker AMJ, Mesker WE, van Velzen MCM, Tanke HJ, Feichtinger J, Ploem JS. Preparation of monolayer smears from paraffin-embedded tissue for image cytometry. Cytometry 1985;6:268-72.
106. Van Driel-Kulker AMJ, Strohmeier R, Naujoks H, Ploem JS. System evaluation of LEYTAS in cervical cancer screening. Anal Cell Pathol 1989;1:266-70.
107. Van Driel-Kulker AMJ, Ploem-Zaaijer JJ. Image cytometry in automated cervix screening. Anal Cell Pathol 1989;1:73-77.
108. Van Ranst M, Kaplan JB, Burk RD. Phylogenetic classification of human papillomaviruses; correlation with clinical manifestation. J Gen Virol 1992;73:2653-60.
109. Villa Santa U. Diagnosis and prognosis of cervical dysplasia. Obstet Gynecol 1971;38:811-6.
110. Wagner D, Sprenger E, Merkle D. Cytometric studies in supicious cervical smears. Acta Cytol 1976;366-71.
111. Walboomers JMM, Roda de Husman AM, Brule van den AJC, Snijders PJF, Meijer CJLM. Detection of genital human papillomavirus: critical review of methods and prevalence studies in realtion to cervical cancer. In: P.L. Stern and M.A. Stanley (eds), Human papillomaviruses and cervical cancer. Biology and immunology, pp 69-71, Oxford University Press, Oxford, New York, Tokyo (1994).
112. Wakonig-Vaartaja R, Kirkland JA. A correlated chromosomal and histopathologic study of pre-invasive lesions of the cervix. Cancer 1965;18:1101-12.
113. Watts KC, Campion MJ, Blance-Butler E, Jenkins D, Singer A, Husain OAN. Quantitive deoxyribonucleic acid analysis of patients with mild cervical atypia: A potentialy malignant lesion? Obstet Gynecol 1987;70:205-7.
114. Weber JE, bartels PH, Bartels HG, Bibbo M. Discrimation of DNA ploidy patterns by order sstatistics. Anal Quant Cytol Histol 1986;9:60-8.
115. Werstro RP, Liblit R, Koss LG. Flow cytometric DNA analysis of human solid tumors: A review of the interpretations of DNA histograms. Hum Pathol 1991;22:1085-98.
116. Wied GL, Bibbo M, Dytch HE, Bartels PH. Computer grading of cervical intraepithelial neoplastic lesions. I. Cytologic indices. Anal Quant Cytol Histol 1984;7:52-60.
117. Wied GL, Bartels PH, Bibbo M, Dytch HE. Image analysis in quantitative cytopathology and histopathology. Human Pathol 1989;20:549-71.
118. Winkler B, Crum C, Fujii T, Ferenzy A, Boon M, Braun L, Lancaster WD, Richart RM. Koilocytotic lesions of the cervix. The relationship of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 1984;53:1081-7.
119. Zuckman MH, Williams G, Levin HS. Mitosis counting in seminoma: an exercise of questionable significance. Hum Pathol 1988;19:329-35.

CHAPTER 2

ATYPICAL MITOTIC FIGURES AND THE MITOTIC INDEX IN CERVICAL INTRAEPITHELIAL NEOPLASIA

Summary

We surveyed cervical intraepithelial neoplasia (CIN) to quantify the proliferation rate and the presence of normal and atypical mitotic figures. In the cervical tissue specimens of 127 women with CIN, the area with the highest cell proliferation was identified and at that site, the proliferation rate was assessed by calculating the mitotic index (MI). Lesions with an MI <2 were not considered further. In the area with the highest proliferation rate, 228 mitoses were classified into one of the following groups: a) normal mitotic figures (NMFs), b) lag type mitoses (LTMs), comprising three group metaphases (3GMs), two group metaphases (2GMs) and other lag type mitoses (OLTMs), c) multipolar mitoses (MPMs), comprising tripolar mitoses (3PMs) and quadripolar mitoses (4PMs), and d) other atypical mitotic figures (OAMFs).

The median value of the MI increased significantly from 3 in CIN I through 4 in CIN II to 9 in CIN III ($p<0.001$). The occurrence of the different LTMs was mutually correlated. The frequency of LTMs increased significantly with increasing CIN grade ($p<0.001$), whereas the frequency of NMFs decreased significantly with increasing CIN grade ($p<0.001$). The frequency of OAMFs was not related with CIN grade ($P=0.94$). MPMs were present in low numbers in a minority of the lesions.

Spearman's rank correlation coefficient (with 95% confidence limits) between the MI and the number of LTMs, OAMFs and NMFs was 0.66 (0.53; 0.75), -0.14 (-0.32; 0.05) and -0.51 (-0.63; -0.35), respectively.

We conclude that increasing CIN grade is associated with increasing MI, increasing numbers of LTMs and decreasing numbers of NMFs. MPMs are very rare events in CIN. The abundant presence of OAMFs seems to be independent of CIN grade and MI.

Introduction

The presence of abnormal mitotic figures (AMFs) and an increased proliferation rate support a diagnosis of CIN in equivocal cases [1,5,15]. Although AMFs are considered to be characteristic features of CIN and are of diagnostic importance, little is known of the prevalence and interrelationships of specific AMFs in this histological entity. Therefore, we surveyed the mitotic figures and the proliferation rate of CIN lesions. More specifically, we investigated: 1) the proliferation rate of CIN lesions, 2) the spec-

trum of mitotic figures in CIN, and 3) the relationship between the proliferation rate and the presence of particular atypical mitotic figures in CIN. The study was performed as a cross-sectional study on newly diagnosed patients with CIN.

Materials and methods

Patients

Patients were recruited from the colposcopy clinic of the Department of Gynaecology, University Hospital, Groningen. They were eligible for participation in the study if 1) it was their first referral with an abnormal cervical cytology report which indicated intraepithelial neoplasia; 2) they were subsequently diagnosed with CIN grade I, II or III; 3) there were no abnormalities in the cylindrical epithelium and 4) they were not pregnant. From 1 September 1988 to 1 May 1991, 148 consecutive patients were eligible for the study. Twenty-one cases were excluded because of morphologically unsatisfactory material (n=10) or an insufficient amount of material (n=11), which left 127 patients.

Routine processing of the tissue specimens

If CIN was diagnosed in the biopsies, the whole transformation zone was excised about 6 to 10 weeks later by either loop electrosection or cold knife conization. Both treatment techniques removed the entire lesion for morphological examination. The diathermy loop was used if the entire squamocolumnar junction could be seen and it did not extend up into the canal for more than 5 mm, measured from the anatomical os externum. Details of the technique have been described previously[4]. The tissue was fixed immediately in buffered formalin 8%, pH 7.42. After paraffin embedding, at least four sections of 4 µm thickness were cut in an anterior-posterior direction and processed routinely for HE staining. Cold knife conization was performed when the neosquamocolumnar junction extended up into the endocervical canal for more than 5 mm, measured from the anatomical os externum. For histopathological analysis, the excised cone was incised at the 12 o'clock position, stretched, fixed in formalin and embedded in paraffin. At each hour position, a section of 4 µm thickness was cut and the 12 sections obtained were processed routinely for HE staining.

The histological diagnosis of CIN according to the WHO criteria[12] was made independently by both pathologists (HH and WJLMP). If there was a discrepancy in the diagnosis, the specimens were re-examined by both the pathologists together and a consensus diagnosis was made. The pathologists were unaware of the mitosis count results.

Examination of mitoses

The microscopical examinations were performed with a Leitz dialux 20 EB microscope with a 40x npl fluotar Leitz objective (n.a. 0.70) and a 10 x widefield periplan ocular piece. The analysis of the mitotic figures was done by one person (AMvL).

For the purpose of this study, new sections of 4 µm thickness were made from all the tissue blocks, in order to achieve uniform quality of the study material. These sections were used to identify the area with the highest mitotic index (MI). This area could be localized in one of the biopsies or in the electrosectioned slice or in the cold knife conus. The MI was assessed by counting the number of mitoses per 1000 nuclei from basal to proximal through the epithelial layer by using a grid. We also assessed the MI on the basis of extended counting and this estimate of the MI will be referred to as MIext. The MIext was assessed to analyse the correlation between an MI per 1000 nuclei and an MI per (approximately) 10.000 nuclei. For the assessment of MIext, 18 additional step sections were cut from the tissue block which contained the area with the highest MI. Every second section was taken for further analysis to ensure that none of the mitoses was considered twice. In nine step sections, the MI was assessed by counting the number of mitoses in an area of equal size as the area which contained 1000 nuclei in the first section. The MIext was defined as the mean value of the MI as estimated in the first section and 9 additional step sections.

Mitotic figures were defined as figures without a nuclear membrane, which indicated that the cell had passed the prophase, and clear hairy extensions of nuclear material should be present. Pycnotic nuclei or nuclei with basophilic cytoplasm were not considered[16]. Mitoses were classified into normal and atypical mitoses. Normal mitotic figures (NMFs) were classified according to the definitions given by Rubio[13]. Atypical mitotic figures (AMFs) were defined as mitoses without the typical aspect of normal mitoses. We classified the AMFs into the following categories:

1. Lag type mitoses (LTMs): figures with nonattached condensed chromatin in the area of the mitotic figure. These were subdivided into
   a. two group metaphases (2GMs): metaphases with nonattached condensed chromatin at one polar side;
   b. three group metaphases (3GMs): metaphases with nonattached condensed chromatin at equidistant positions at the two polar sides;
   c. other lag type mitoses (OLTMs): lag type mitoses without the configuration of a 2GM or 3GM.
2. Multipolar mitoses (MPMs): metaphases with an abnormal configuration of the equatorial plate; the chromosomes were located along several radiar axes. These figures were subdivided into
   a. tripolar mitoses (3PMs): metaphases with three radiar axes;
   b. quadripolar mitoses (4PMs): metaphases with four radiar axes;
3. other atypical mitotic figures (OAMFs): atypical mitoses with a morphological appearance which was not reconcilable with one of the abovementioned classes. The OAMFs included ring mitoses, asymmetrical mitoses, dispersed mitoses, etc.

The analysis of mitotic figures was performed on the first section from the area with the highest visual number of mitoses. On arbitrary grounds, we wanted to be 99% sure that any specific AMF was detected if its frequency among the mitoses in a lesion was actually 2% or more. Statistically, the number n of mitoses had to be so large that the chance $P(n)$ of not observing any specific AMF among n mitoses selected at random from a tissue specimen with a fraction p of that particular AMF was less than α. From the formula

$$P(n) = (1-p)^n \leq \alpha$$

it followed that

$$n \geq \ln(\alpha) / \ln(1-p).$$

For $\alpha = 0.01$ and $p = 0.02$, we calculated $n \geq 228$. If the selected area in the first section was too small to count 228 mitoses, we used additional odd-numbered step sections (which were made for the assessment of MIext). If the MI was 1 or less, the mitotic figu-

res were not analysed in the respective lesion because it was practically impossible to count 228 mitoses.

Statistical analysis

The Kruskal-Wallis one-way analysis of variance was used to examine whether there was a significant difference in the distribution of current age or the mitotic index between the three groups of patients with CIN grade I, II and III. The Spearman's rank correlation coefficient was used to express the relationships between the MI and MIext, between the frequency of various mitotic figures, or between the frequency of mitotic figures and the MI. The statistical and graphical procedures were performed with the SYSTAT software package[18]. The 95% confidence interval of Spearman's rank correlation coefficient was obtained with the CIA (Confidence Interval Analysis) software package[9]. P values of less than or equal to 0.05 were considered to be significant.

Results

Patients

In the group of 127 patients, the histological diagnoses were CIN I (n=15), CIN II (n=28) and CIN III (n=84). The age of the patients ranged from 20 to 66 years, with a mean age of 34.9 (SD 7.4) years. The mean ages of the patients with CIN I, CIN II and CIN III was 32.2 (SD 7.0) years, 35.0 (SD 7.5) years and 35.4 (SD 7.4) years, respectively. There was no difference in the age distribution between the three CIN grades (p=0.30; Kruskal-Wallis one way analysis of variance).

The distribution of the MI and the MIext in CIN

The MI in the 127 lesions ranged from 0 to 51, with a median value of 8. The median values (and the range of the values) of the MI among the CIN I, CIN II and CIN III lesions were 3 (0-8), 4 (1-29) and 9 (1-51), respectively. The distribution of the MI per CIN grade is presented graphically in Figure I. There was a statistically significant dif-

ference in the mitotic index between the three CIN grades ($p<0.001$, Kruskal-Wallis one-way analysis of variance).

The MIext ranged from 0.1 to 43.1 with a median value of 6.5. Spearman's rank correlation coefficient between the MI and the MIext was 0.92 (95% confidence limits 0.88 and 0.94). Out of the 108 cases which showed an MI ≥2, the value of the MIext was <2 in 1 patient. On the other hand, out of the 19 cases which had a MI <2, the MIext was ≥2 (i.e. 2.0, 2.3 and 2.7) in 3 patients. We conclude that the estimates of the MI and the MIext are very similar. We will use the MI in the further statistical analysis.

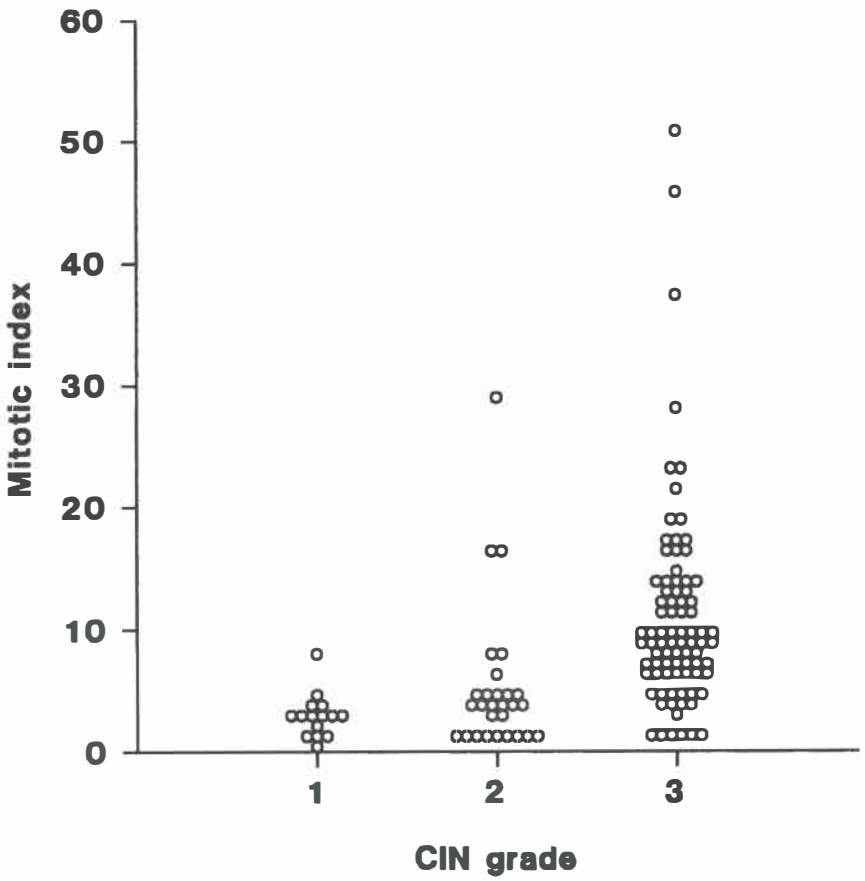


Figure I: Distribution of the mitotic index in relation to the CIN grade (n=127)

The AMF types in CIN

From the total group of 127 patients, 19 (15%) showed a MI <2. In this group of 19 patients, the histological diagnoses were CIN I (n=4), CIN II (n=9) and CIN III (n=6). These 19 patients were not included in the further analysis. We performed a quantitative analysis on the occurrence of 3GM, 2GM, OLTM, 3PM, 4PM, OAMF and NMF in all three CIN grades. The results are shown in Table I.

The 3GM did not occur in CIN I lesions. As compared to the group of CIN II lesions, the group of CIN III lesions showed a higher percentage of 3GM-positive lesions, as well as a higher number of 3GMs per 228 mitotic figures in a lesion. The 3GM remains, however, an infrequent finding: a total of 31 (44%) out of the 71 3GM-positive lesions showed ≤4 3GMs per 228 mitoses, which means a frequency of less than 2%. The 2GM was found in nearly all of the lesions, including the CIN I lesions. Its frequency among the mitotic figures increased with increasing severity of the CIN lesion, from a median value of 3 2GMs per 228 mitoses in CIN I to 22 2GMs per 228 mitoses in CIN III. OLTMs were found in all of the lesions with the exception of one CIN I lesion and the frequency increased also with increasing severity of the CIN lesion. The frequencies of OLTM and 2GM in CIN lesions were very similar.

We subsequently analysed the relationships between the specific lag type mitoses. Spearman's rank correlation coefficient between the occurrence of 3GM and 2GM was 0.83 with a 95% confidence interval from 0.76 to 0.88. All of the lesions which showed 3GM also showed 2GM. In 30 cases, 2GM was present without the simultaneous presence of 3GM. Spearman's rank correlation coefficient between the occurrence of 3GM and OLTM was 0.70 with a 95% confidence interval from 0.59 to 0.79. We conclude that the specific lag type mitotic figures are strongly correlated with each other.

Only 24 (22%) out of the 108 lesions displayed multipolar mitoses (MPMs), i.e. 3PMs and/or 4PMs. In all of these 24 cases, the total number of multipolar mitoses did not exceed 3 per 228 mitoses.

The group of OAMFs comprised the atypical mitotic figures which could not clearly be delineated as a LTM or MPM. The median number of OAMFs per 228 mitoses ranged from 112 in CIN I lesions to 104 in CIN III lesions.

Table I. Distribution of atypical and normal mitotic figures in the three CIN grades. Mitotic figures were counted among 228 mitoses in lesions which showed a mitotic index ≥2.

		CIN I	CIN II	CIN III
Total	number	11	19	78
3GM	n	0	7	64
	median	3	6	
	range	1-7	1-17	
2GM	n	10	14	77
	median	3	12	22
	range	1-4	1-34	2-61
OLTM	n	10	19	78
	median	3	10	21
	range	1-7	2-42	2-69
3PM	n	2	6	14
	median	1.5	1	1
	range	1-2	1-2	1-3
4PM	n	0	1	7
	median	1		
	range	1	1-2	
OAMF	n	11	19	78
	median	112	109	104
	range	73-163	61-160	41-196
NMF	n	11	19	78
	median	111	94	70
	range	59-151	21-150	12-136

n=number of positive cases;

median and range refer to the the number of figures within the group of lesions which showed that particular figure

ERRATUM

Ondanks herhaalde controle op de zetting zijn helaas in de laatste fase een aantal tabellen niet juist afgedrukt.

ad blz 38

Table I. Distribution of atypical and normal mitotic figures in the three CIN grades. Mitotic figures were counted among 228 mitoses in lesions which showed a mitotic index ≥2.

		CIN I	CIN II	CIN III
Total number		11	19	78
3GM				
	n	0	7	64
	median		3	6
	range		1-7	1-17
2GM				
	n	10	14	77
	median	3	12	22
	range	1-4	1-34	2-61
OLTM				
	n	10	19	78
	median	3	10	21
	range	1-7	2-42	2-69
3PM				
	n	2	6	14
	median	1.5	1	1
	range	1-2	1-2	1-3
4PM				
	n	0	1	7
	median			1
	range		1	1-2
OAMF				
	n	11	19	78
	median	112	109	104
	range	73-163	61-160	41-196
NMF				
	n	11	19	78
	median	111	94	70
	range	59-151	21-150	12-136

n=number of positive cases;

median and range refer to the the number of figures within the group of lesions which showed that particular figure

The median number of normal mitotic figures (NMFs) per 228 mitoses ranged from 111 in CIN I to 70 in CIN III.

Figure II shows graphically the distribution of LTMs, OAMFs en NMFs per CIN grade. The frequency of LTMs increased significantly with increasing CIN grade ($P<0.001$; Kruskal-Wallis one way analysis of variance). The frequency of OAMFs was not related with CIN grade ($P=0.94$; Kruskal-Wallis one way analysis of variance). The frequency of the NMFs decreased significantly with increasing CIN grade ($p<0.001$; Kruskal-Wallis one-way analysis of variance. Because of small numbers, the frequency distribution of MPMs per CIN grade were not included in this figure.

The relationship between the MI and AMF types in CIN

The 3GM was found at MI values of 4 or higher. At MI values of 10 or higher, the 3GM was absent in only 3 (7%) out of the 43 cases. The presence of the 3GM in relation to the mitotic index is depicted in Figure III. The 2GM was found in a proportion of the lesions with MI values from 2 through 5, and this figure was always present at MI values of 6 or higher. As stated above, 2GM was present without the simultaneous occurrence of 3GM in 30 lesions, all of which had a comparatively low mitotic index.

When we considered the lag type mitoses as a group, Spearman's rank correlation coefficient between the number of LTMs and the MI was 0.66 (95% confidence limits 0.53 and 0.75). We conclude that the frequency of lag type mitoses increases significantly with increasing CIN grade.

Spearman's rank correlation coefficient between the MI and the number of OAMFs was -0.14 (95% confidence limits -0.32 and 0.05), which means that these variables are not significantly associated. However, we did find a significant negative relationship between the MI and the presence of NMFs (Spearman's rank correlation coefficient between these variables was -0.51 with -0.63 and -0.35 as the 95% confidence limits). It can be argued that CIN grade confounds the association between the MI and the presence of AMFs, because the MI is also associated with CIN grade. We therefore performed the analysis within the group of CIN III lesions only. The correlation coefficients are somewhat lower, but the conclusions are essentially unchanged: Spearman's rank correlation coefficients (with 95% confidence limits) between the MI and the number of

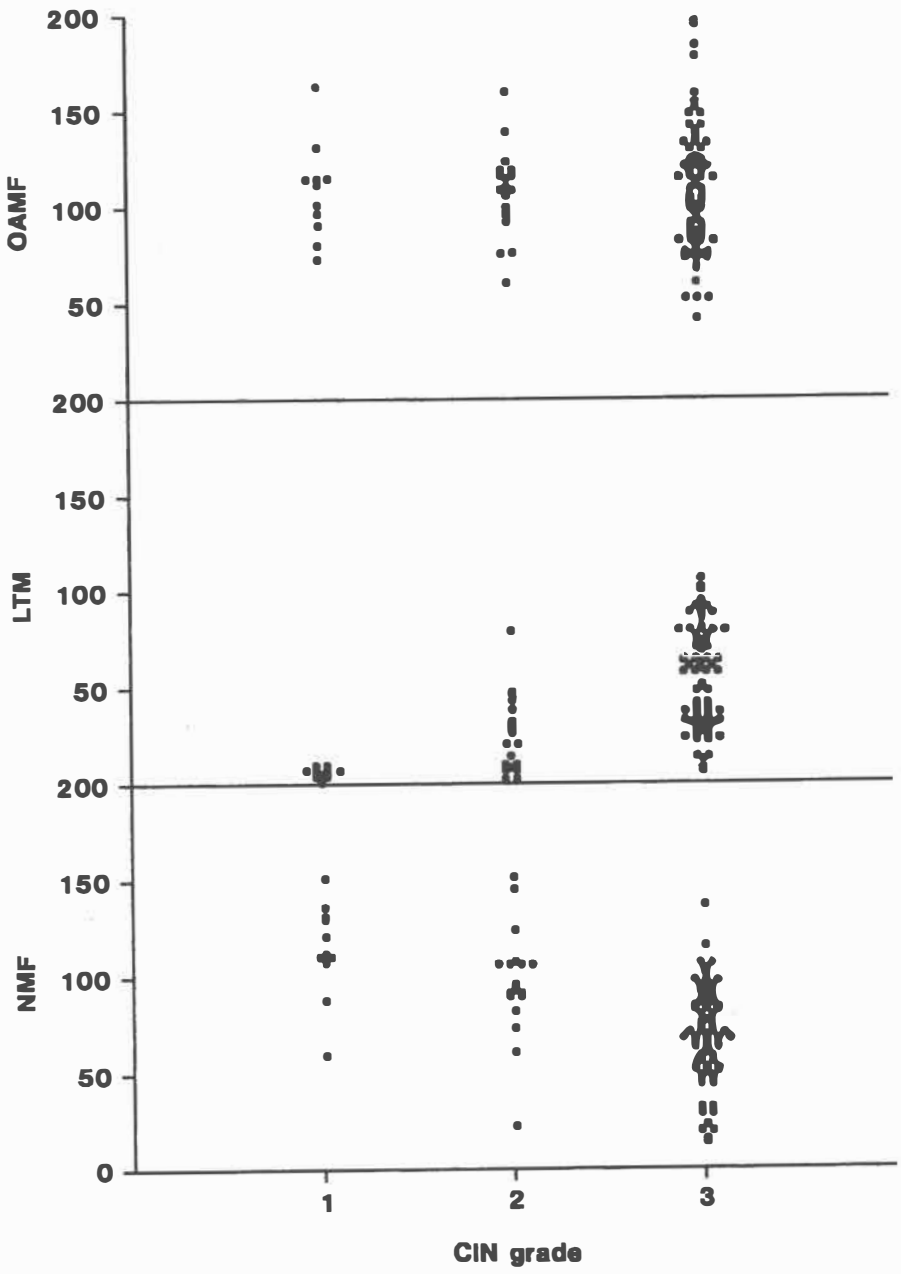


Figure II: The frequency of LTMs, OAMFs and NMFs among 228 mitoses in relation to the CIN grade (n=108; cases with MI <2 excluded)

LTMs, OAMFs and NMFs in CIN III lesions were 0.44 (0.24;0.60), -0.17 (-0.37;0.06) and -0.24 (-0.44;-0.02), respectively. Because of small numbers, the MPMs were not considered in this part of the analysis.

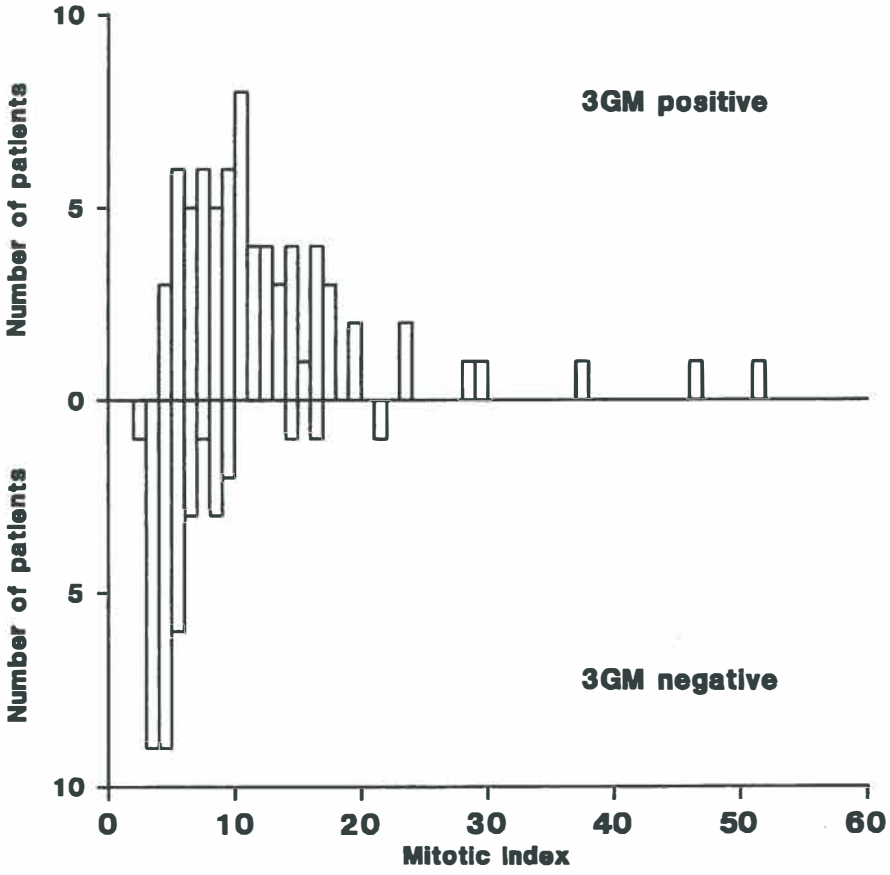


Figure III: Distribution of the mitotic index in relation to the presence of 3GM among 228 mitoses in CIN lesions (n=108; cases with MI <2 excluded)

Discussion

In the literature, the proliferation rate of a lesion is commonly expressed as the number of mitoses per 10 high power fields. This method has the disadvantage that the outcome depends on the cell size and the square area of the high power field[7]. We preferred to assess the cell proliferation rate by counting the number of mitoses per 1000 nuclei (i.e. the mitotic index or MI) in the area with visually the highest number of mitoses. We demonstrated that this estimate did not substantially differ from the result by counting the number of mitoses per 10.000 nuclei in stepsections from this area.

In this study, a standard number of mitoses were counted in the lesions from a large group of consecutively enrolled women. In each lesion, 228 mitoses were counted in sections which were cut and stained at one laboratory (Laboratory of Pathology, Winschoten); this condition produced slides of constant quality. To minimize intra-individual variation in the examination of mitoses, only the well-defined and clearly recognizable mitotic figures were considered. We adhered to the rules for mitosis counting and any technically unsatisfactory specimens were excluded from the analysis[2].

We encountered two reports on mitosis counting in CIN[5,15]. Both authors found an increased proliferation with increasing CIN grade, which is in agreement with our findings.

Very few reports have appeared on the quantitative analysis of different types of mitotic figures in cervical lesions. No meaningful comparison is possible between our results and those of others, because the selection of patients and counting procedures used were completely different. We demonstrated a strong association between the occurrence of LTMs and CIN grade. We found 3GM only in CIN II and CIN III lesions, which confirms the data of other investigators[6]. The 3GM is the best studied mitotic figure of the lag type and its presence seems to be characteristic of neoplasia. In chemically induced lesions of the uterine cervix in mice, 3GM was found in carcinoma in situ and invasive carcinoma, but was absent in dysplastic and inflammatory lesions[14]. In micro-invasive cervical carcinomas of humans, the intraepithelial part contained 3GM in 83%-93% of the cases[6,10]. In an image cytometry study, 3GM proved to be a better indicator of the presence of a large number of nonpolyploid high ploid cells in CIN than the traditional histological CIN classification[11]. As large numbers of these cells are related to aneuploidy[17], 3GM can be regarded as an indicator of aneuploidy, which is an esta-

blished determinant of progressive CIN lesions[8,3]. In a series of koilocytotic lesions, the presence of 3GM was also confined to the lesions which were aneuploid[19].

We demonstrated that MPMs are seldomly found in CIN lesions. The OAMFs were present in large numbers in all of the CIN lesions, but their frequency was not associated with the CIN grade. The increase of LTMs with increasing CIN grade seems to occur at the cost of the NMFs.

In the present study, we have described quantitatively the proliferation rate and the spectrum of AMFs in CIN. To study their possible diagnostic importance, particularly regarding the LTMs, we will perform a survey of the intraepithelial part of micro-invasive carcinomas and non-neoplastic cervical epithelial changes for the mitotic index and the mitotic figures.

References

1. Anderson MC, Brown CL, Buckley CH, Fox H, Jenkins D, Lowe DG, Manners BTB, Melcher DH, Robertson AJ, Mells M (1991) Current views on cervical intraepithelial neoplasia. J Clin Pathol 44:969-978.
2. Baak JPA (1990) Mitosis counting in tumors. Hum Path 21:683-685. (Editorial)
3. Bibbo M, Dytch HE, Alenghat E, Bartels PH, Wied GL (1989) DNA ploidy profiles as prognostic indicators in CIN lesions. Am J Clin Path 92:261-265.
4. Burger MPM, Hollema H (1993) The reliability of the histologic diagnosis in colposcopically directed biopsies. A plea for LETZ. Int J Gynecol Cancer 3:385-390.
5. Chi CH, Rubio CA, Lagerlof B (1977) The frequency and distribution of mitotic figures in dysplasia and carcinoma in situ. Cancer 39:1218-1223.
6. Claas ECJ, Quint WGV, Pieters WJLM, Burger MPM, Oosterhuis JW, Lindeman J (1992) Human Papillomavirus and the three group metaphase as markers of an increased risk for the development of cervical carcinoma. Am J Path 140:497-502.
7. Ellis PSJ, Whitehead R (1981) Mitosis counting. A need for reappraisal. Human Pathol 12:3-4.
8. Fu YS, Reagan JW, Richart RM (1981) Definition of precursors. Gyn Oncol 12:S220-231.
9. Gardner MJ, Gardner SB, Winter PD (1991) Confidence Interval Analysis (CIA) Microcomputer program. London: British Medical Journal.
10. Mourits MJE, Pieters WJLM, Hollema H, Burger MPM (1992) Three group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 167:591-595.
11. Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW (1992) The three group metaphase is a morphological indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 14:227-232.
12. Poulsen HE, Taylor CW, Sobin LH (1975) Histological typing of female genital tract tumours. Geneva: World Health Organization.
13. Rubio CA (1991) Atypical mitosis in colorectal adenomas. Path Res Pract 187:508-513.
14. Scarpelli DG, Von Haam E (1957) A study of mitosis in cervical epithelium during experimental inflammation and carcinogenesis. Cancer 17:880-885.
15. Tanaka T (1986) Proliferative activity in dysplasia, carcinoma in situ and microinvasive carcinoma of the uterine cervix. Path Res Pract 181:531-539.
16. Van Diest PJ, Baak JPA, Matze-Cok P, Wisse-Brekelmans ECM, Van Galen CM, Kurver PHJ, Bellot SM, Fijnheer J, Van Gorp LHM, Kwee WS, Los J, Peterse JL, Ruitenberg HM, Schapers RFM, Schipper MEI, Somsen JG, Willig AWPM, Ariens ATh (1992) Reproducibility of mitosis counting in 2,469 breast cancer specimens. Results from the multicenter morphometric mammary carcinoma project. Human Pathol 23:603-607.
17. Van Driel-Kulker AMJ (1986) Automated image analysis applied to the diagnosis of cervical cancer. Grenoble: University of Grenoble, p 89. Dissertation.
18. Wilkinson (1990) SYSTAT: The System for Statistics. Evanston, IL: SYSTAT Inc.
19. Winkler B, Crum C, Fujii T, Ferenzy A, Boon M, Braun L, Lancaster WD, Richart RM (1984) Koilocytotic lesions of the cervix. The relationship of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 53:1081-1087.

CHAPTER 3

ATYPICAL MITOTIC FIGURES AND THE MITOTIC INDEX IN MICROINVASIVE CARCINOMAS AND IN NON-NEOPLASTIC CHANGES OF THE UTERINE CERVIX

Summary

This study sought to show whether an increased mitotic index (MI; i.e. the number of mitoses among 1000 nuclei) and the occurrence of particular atypical mitotic figures (AMFs) are characteristic of cervical squamous malignancy.

The study group consisted of 24 cervical tissue specimens with microinvasive carcinoma (MIC). The intraepithelial and invasive parts were considered separately. The control group consisted of 82 cervical tissue specimens without neoplasia which were derived from two sources: 31 women were biopsied because of an abnormal cervical cytology report but no neoplasia was found histologically and 51 women underwent an hysterectomy for non-cervical uterine disease. Mitoses were only classified in proliferative lesions (MI ≥2). The mitoses were classified into normal mitotic figures (NMFs), lag type mitoses (LTMs), multipolar mitoses (MPMs) and other atypical mitotic figures (OAMFs). Particular attention was given to the two group metaphase (2GM), a member of the LTMs. In order to be 99% confident to detect any particular AMF which actually made up at least 2% of the mitoses, 228 mitoses were classified in each area under study.

In the intraepithelial part of MIC, the MI ranged from 3 to 17 with a median value of 7. All of these areas showed the two-group metaphase (2GM) among 228 mitoses. In the invasive areas, the MI ranged from 3 to 13 with a median value of 7.5 and all of these areas showed 2GMs. The frequency of the 2GM was significantly less in the invasive area than in the intraepithelial part ($p=0.01$). MPMs were seldomly seen in MIC, whereas NMFs and OAMFs occurred frequently.

In the group of 31 women with abnormal smears but with non-neoplastic cervical changes, the MI ranged from 0 to 4 with a median value of 1. Mitoses were classified in 8 (26%) of the 31 specimens. The 2GM was found in 2 mature metaplasias with an MI of 2. One of the specimens showed basal cell hyperplasia with a MI of 3 and 3 2GMs among 228 mitoses. In the group of 51 patients with non-cervical uterine disease, the MI ranged from 0 to 5 with a median value of 1. Mitoses were classified in 12 (24%) of the 51 specimens. A single 2GM among 228 classified mitoses was found in 2 mature metaplasias with a MI value of 2. MPMs mitoses were seldomly seen in the non-neoplastic epithelia, whereas NMFs and OAMFs occurred frequently.

We conclude that the intraepithelial area in specimens with MIC is characterized by a minimum MI value of 3 and the presence of 2GM among 228 mitoses. This combina-

tion of features does not occur in non-neoplastic epithelium, apart from an exceptional case of basal cell hyperplasia. LTMs will indicate malignancy if they can be found easily. However, the presence of LTMs is not pathognomonic of malignancy, since an occasional LTM can be found in non-neoplastic epithelium. Mitotic figures other than LTMs do not seem to have any diagnostic importance.

Introduction

Cervical intraepithelial neoplasia (CIN) are characterized by increased mitotic activity and the occurrence of atypical mitotic figures[1,2,14]. In a previous study on CIN lesions, we have demonstrated that both the mitotic index (MI, i.e. the number of mitoses among 1000 nuclei) and the relative number of abnormal mitotic figures of the lag type (LTMs) increase with increasing CIN grade[16]. The three group metaphase (3GM), the best studied member of the LTMs, is frequently found in microinvasive carcinoma[8], and its presence is associated with aneuploidy in CIN lesions[17] and with a large number of high-ploidy cells in CIN lesions[17]. Another well-defined member of the LTMs is the 2GM, which occurs more frequently than the 3GM.

In the present study, we analysed whether an increased MI and the occurrence of particular mitotic figures are characteristic of malignancy. For that purpose, we performed a survey of the MI and the presence of atypical mitotic figures in malignant cervical squamous epithelium and in cervical squamous epithelium without CIN. Malignant epithelium was studied in specimens with micro-invasive carcinoma (MIC). The intraepithelial and the invasive part of MIC were considered separately. Cervical squamous epithelium without CIN was studied in two different patient groups.

Materials and methods

Patients

The patients with cervical malignant squamous epithelial lesions consisted of women with MIC of the cervix. The histological reports of 47 patients diagnosed between 1 January 1988 and 1 September 1992 were collected from the routine files of

the pathological laboratories of 10 hospitals in the north of the Netherlands. All of the slides were reviewed by one pathologist (WJLMP). MIC was defined as a lesion with an invasion depth of ≤5 mm. In 13 (28%) out of the 47 cases, the presence of invasion was doubtful and these cases were excluded from the study. The surface epithelium above or adjacent to the invasive area in one specimen (2%) could not be classified as CIN, so this case was also excluded. Eight (17%) out of the 47 cases were excluded because of morphologically unsatisfactory material and in one case (2%) there was no surface epithelium above or adjacent to the area of invasion. Therefore, this study was performed on the histological specimens of 24 patients. The age of the patients ranged from 27 to 58 years with a mean of 39.3 (SD 9.4) years. None of the patients were included in our previous study on micro-invasive carcinoma[8].

We studied two groups of patients with non-neoplastic cervical squamous epithelium.

The first group consisted of patients with a reported cervical cytological abnormality but in whom no neoplasia was found in the biopsies. They were recruited from the outpatients clinic of the Department of Gynaecology of the University Hospital Groningen. From 1 September 1988 up to 1 September 1992, 34 consecutive patients were eligible for participation in this study group. Three cases were excluded because of unsatisfactory histological material, which left 31 patients. The ages of the patients ranged from 19 to 65 years with a mean of 36.1 (SD 10.7) years.

The second group consisted of patients with normal cytological and histological findings in the cervix. The patients underwent a hysterecomy for reasons not related to an abnormal cervical smear or cervical neoplasia. Their reports were collected from the routine files of the Department of Pathology of the University Hospital Groningen. Postmenopausal women were not considered. From 1 January 1992 up to 1 November 1992, 53 patients were eligible for participation in this group of the study. Two cases were excluded because of morphologically unsatisfactory material, which left 51 patients. The ages of the patients ranged 22 to 59 years with a mean of 41 (SD 7.4) years.

Methods

In the group of women with MIC, the histological specimens were cones (n=15), electrosectioned tissue strips (n=3), uteri (n=2) and both biopsies and cones (n=4). In the

specimens, the intraepithelial part is defined as CIN lying adjacent to or above the invasive part. All intraepithelial parts were classified as high grade lesion. In the case of small MICs, the overlying intraepithelial part could be discriminated from the invasive part by the virtual reconstruction of the course of the basement membrane. In the case of larger MICs, whether or not accompanied by ulceration, the nearest intraepithelial lesion with a distinct basement membrane was held to be the intraepithelial part.

The tissue specimens from the women with cytological abnormalities but in whom no neoplasia was found, comprised biopsies and one cervical cone. Our protocol for the diagnostic work-up of cervical cytological abnormalities prescribed that a biopsy had to be taken from every cervical site with distinct colposcopic features. If no colposcopical abnormality was seen, a biopsy was taken at the 12 o'clock and at the 6 o'clock position. The biopsies were fixed in buffered formalin 8%, pH 7.42 and embedded in paraffin. One of the patients underwent a conisation. The excised cone was incised at the 12 o'clock position, stretched, fixed in formalin, cut at each hour position to obtain 12 blocks which were subsequently embedded in paraffin.

The hysterectomy specimens from the second group of patients with non-neoplastic cervical epithelium were fixed in buffered formalin 8%, pH 7.42. The cervix was cut in the 12 to 6 o'clock direction and the two separate blocks, representing the area at the 12 and 6 o'clock position, were embedded in paraffin.

From the original tissue blocks, new 4 µm paraffin sections were cut and H-E stained. These sections were used to identify the area with the highest mitotic index (MI). The MI was assessed by counting the number of mitoses per 1000 nuclei from basal to proximal through the epithelial layer. In the invasive parts of specimens with MIC, the nuclei were counted by using a grid (NGMI, 19 mm).

The analysis of mitotic figures was performed by one person (AMvL) on the same set of sections, starting with the first section from the area with the highest MI. A total of 228 mitoses were classified. The number of 228 mitoses was chosen to be 99% confident that any particular atypical mitotic figure would be detected if that figure actually made up at least 2% of the mitoses[16]. If the selected area in the first section was too small to count 228 mitoses, additional step sections from the same area were made and every second section was taken for further analysis to ensure that none of the mitoses

was considered twice. If the MI was 1 or less, the mitotic figures were not analysed in the respective lesion because it was practically impossible to count 228 mitoses.

Mitotic figures were defined as figures without a nuclear membrane, which indicated that the nucleus had passed the prophase, and with clear hairy extensions of the nuclear material. Pycnotic nuclei or nuclei with basophilic cytoplasm were disregarded[15]. Mitoses were classified into normal and atypical mitoses. Normal mitotic figures (NMF) were assessed in accordance with the definitions given by Rubio[12]. We considered the following atypical mitotic figures: 2GM, 3GM, other lag type mitoses (OLTM), tripolar mitoses (3PM), quadripolar mitoses (4PM) and other atypical mitotic figures (OAMF). The definitions of the atypical mitotic figures are summarized in Table I.

The microscopical examinations were performed with a Leitz dialux 20 EB microscope with a 40x npl fluotar Leitz objective (n.a. 0.70) and a 10x wide-field periplan ocular piece.

Table I. Definitions of atypical mitotic figures

1. Lag type mitoses: mitoses with nonattached condensed chromatin in the area of the mitotic figure. These are subdivided into:
   a. two group metaphases (2GMs): metaphases with nonattached condensed chromatin at one polar side;
   b. three group metaphases (3GM): metaphases with nonattached condensed chromatin at equidistant positions at the two polar sides;
   c. other lag type mitoses (OLTM), lag type mitoses without the configuration of a 2GM or 3GM.
2. Multipolar mitoses: metaphases with an abnormal configuration of the equatorial plate; the chromosomes are located along several radiar axes. These figures are subdivided into:
   a. tripolar mitoses (3PM): metaphases with three radiar axes;
   b. quadripolar mitoses (4PM): metaphases with four radiar axes;
3. Other atypical mitotic figures (OAMF): atypical mitoses with a morphological appearance which was not reconcilable with one of the above-mentioned classes. The OAMFs included ring mitoses, asymmetrical mitoses, dispersed mitoses, etc.

Statistical analysis

The Wilcoxon test for matched pairs was used to examine whether there was a significant difference in the distribution of the MI or the count of specific atypical mitotic figures between two areas in specimens with MIC. In this case, P-values of less than or equal to 0.01 were considered to be significant. The value 0.01, instead of the more commonly used 0.05, resulted from the correction according to Bonferroni which should be used in case of multiple comparisons[7].

Results

Specimens with MIC

Firstly, we analysed the group of patients with MIC. Table II shows the details concerning the occurrence of the various mitotic figures in the respective areas.

All of the 24 intraepithelial areas were classified histologically as high grade lesions. In these areas, the MI ranged from 3 to 17 with a median value of 7. The 3GM was found in 20 (83%) out of the 24 specimens. The 2GM was found in all of the intraepithelial areas, as were the OLTM, OAMF and NMF. Multipolar mitoses were found in low numbers in a minority of the intraepithelial areas.

The invasive area of 16 out of the 24 specimens was analysed. The other 8 specimens were excluded because 4 areas showed less than 1000 nuclei and 4 additional areas (which showed MI values of 3, 3, 6 and 31, respectively) were not large enough to classify 228 mitoses. In the invasive areas, the MI ranged from 3 to 13 with a median value of 7.5. The 3GM was found in 11 (69%) out of the 16 cases. The 2GM was found in all of the invasive areas, as were the OLTM, OAMF and NMF. Multipolar mitoses were found in low numbers in a minority of the invasive areas.

In the same group of 16 cases, we compared the intraepithelial and invasive area with regard to the distribution of the MI and the occurrence of the various mitotic figures. The areas did not differ with regard to the MI ($p=0.36$; Wilcoxon matched-pairs test). The 2GM occurred significantly more often in the intraepithelial area than in the invasive area ($p=0.01$; Wilcoxon matched-pairs test). When the lag type mitoses (2GM, 3GM and OLTM) were considered as a group, the frequency of the lag type mitoses in

Table II. Distribution of mitotic figures in the intraepithelial and invasive areas of specimens which showed microinvasive carcinoma. 228 mitoses were classified

Mitotic figure		Intraepithelial area	Invasive area
Total	number	24	16
2GM	n	24	16
	median	11.5	6
	range	1-43	1-23
3GM	n	20	11
	median	2	2
	range	1-12	1-9
OLTM	n	24	16
	median	11.5	10
	range	5-30	3-20
3PM	n	9	7
	median	1	3
	range	1-8	1-7
4PM	n	2	4
	median	1.5	1.5
	range	1-2	1-4
OAMF	n	24	16
	median	110	120.5
	range	46-161	63-180
NMF	n	24	16
	median	82.5	86
	range	55-161	34-160

n = number of positive cases

median and range refer to the number of mitotic figures within the group of lesions which showed that particular mitotic figure

Table III. Distribution of mitotic figures in the cervix of women with cytological abnormalities indicating neoplasia but in whom no neoplasia was found (group A) and of women with normal cytological and histological findings (group B). The analysis was restricted to women with proliferative (MI ≥2) cervical epithelium (8 out of 31 women from group A and 12 out of 51 from group B). 228 mitoses were classified

Mitotic figure		Group A	Group B
Total number of women with proliferative (MI ≥2) epithelium		8	12
2GM	n	3	2
	median	2	1
	range	1-3	1
3GM	n	0	0
	median		
	range		
OLTM	n	3	7
	median	2	2
	range	1-8	1-3
3PM	n	0	1
	median	1	
	range	1	
4PM	n	0	0
	median		
	range		
OAMF	n	8	12
	median	97	88.5
	range	78-138	53-140
NMF	n	8	12
	median	125	138.5
	range	88-148	85-175

n=number of positive cases

median and range refer to the number of mitotic figures within the group of lesions which showed that particular mitotic figure

Table III. Distribution of mitotic figures in the cervix of women with cytological abnormalities indicating neoplasia but in whom no neoplasia was found (group A) and of women with normal cytological and histological findings (group B). The analysis was restricted to women with proliferative (MI ≥2) cervical epithelium (8 out of 31 women from group A and 12 out of 51 from group B). 228 mitoses were classified

Mitotic figure		Group A	Group B
Total number of women with proliferative (MI ≥2)epithelium		8	12
2GM			
	n	3	2
	median	2	1
	range	1-3	1
3GM			
	n	0	0
	median		
	range		
OLTM			
	n	3	7
	median	2	2
	range	1-8	1-3
3PM			
	n	0	1
	median		1
	range		1
4PM			
	n	0	0
	median		
	range		
OAMF			
	n	8	12
	median	97	88.5
	range	78-138	53-140
NMF			
	n	8	12
	median	125	138.5
	range	88-148	85-175

n=number of positive cases
median and range refer to the number of mitotic figures within the group of lesions which showed that particular mitotic figure

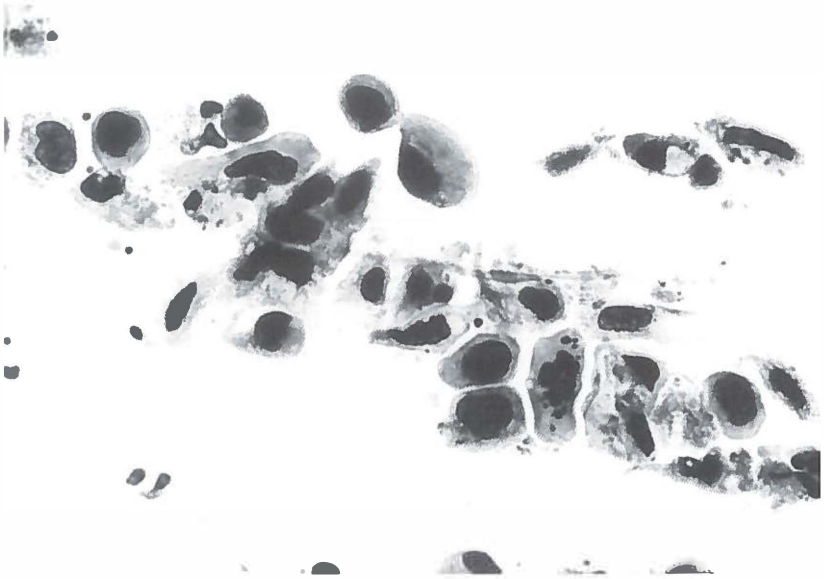
the intraepithelial area was also significantly higher than in the invasive area (p=0.01; Wilcoxon matched-pairs test). The frequency of OAMFs and NMFs did not differ between the two areas.

Specimens without neoplasia

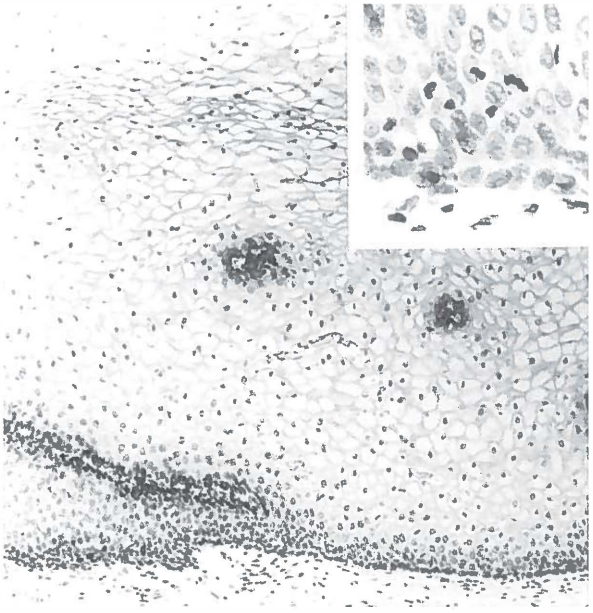
We subsequently analysed the group of patients with cervical biopsies in which no neoplasia was found, albeit the cervical smear indicated dysplasia. The MI ranged from 0 to 4 with a median value of 1. In the specimens of 8 (26%) out of the 31 patients, the lesion was proliferative (MI ≥2). Table III summarizes the results of the classification of 228 mitoses in these 8 specimens. None of the specimens showed the 3GM, whereas the 2GM and the OLTM were both found in 3 specimens. All of the cases demonstrated OAMF and NMF, whereas none of them demonstrated the multipolar mitoses.

In addition, we analysed the group concerning the cervical tissue in the hysterectomy specimens of women with non-cervical uterine diseases. The MI ranged from 0 to 5 with a median value of 1. In the specimens of 12 (24%) out of the 51 patients, the lesion was proliferative (MI ≥2). Table III summarizes the results of the classification of 228 mitoses in these 12 specimens. None of the specimens showed the 3GM. The 2GM was found in 2 of the specimens, while OLTMs were found in 7 of them. All of the specimens showed the OAMF and the NMF. Only one hysterectomy specimen showed a single 3MP.

The results showed that lag type mitoses can be found in non-neoplastic changes of the cervix. The cases which displayed 2GM are presented in more detail. In case no. 1, the patient was seen because of two consecutive abnormal cytology reports indicating mild dysplasia. The biopsies showed metaplasia with chronic inflammation. The MI was 2, and the area concerned showed 1 (0.4%) 2GM, 86 (38%) OAMFs and 141 (62%) NMFs. In case no. 2, the patient had two consecutive Pap smears which indicated mild dysplasia. The biopsies showed mature metaplasia with chronic inflammation. The MI was 2, and the 228 mitoses were classified into 2 (0.9%) 2GMs, 138 (61%) OAMFs and 88(39%) NMFs. In case no. 3, the patient was referred to our hospital because of a cervical smear indicating severe dysplasia. The smear showed atypical cells with hyperchromatic polymorphic nuclei with indistinct cell borders. No dysplasia was found in the



Photograph I: Pap-smear of case no. 3 showing atypical cells with hyperchromatic polymorphic nuclei with indistinct cell borders (HE x 560)



Photograph II: Histological section of the cone of case no. 3 showing non-dysplastic squamous epithelium with minor basal cell hyperplasia (HE x 100). Inset: magnification of base of epithelium showing a lag type mitoses (HE x 400)

colposcopically directed biopsies. The MI was 2, and neither 3GM nor 2GM could be demonstrated among the 228 classified mitoses. Six weeks later a conization was done because of the discrepancy between the cytological diagnosis and the histological findings. Likewise, no dysplasia was found in the cone. The cone showed basal cell hyperplasia with a MI of 3. The 228 mitoses were classified into 3 (1.3%) 2GMs, 1 (0.4%) OLTM, 100 (44%) OAMFs and 124 (54%) NMFs. The cytological and histological findings in this patient are presented in Photograph I-II. In case no. 4, the patient underwent a hysterectomy because of endometriosis and leiomyoma. The cervix showed histologically mature metaplasia without atypia. The MI was 2, and the 228 mitoses in the area concerned were classified into 1 (0.4%) 2GM, 2 (0.8%) OLTMs, 126 (55%) OAMFs and 99(43%) NMFs. In case no. 5, the hysterectomy was done because of leiomyoma. The cervix showed histologically mature metaplasia. The MI was 2. Among the 228 classified mitoses, 1 (0.4%) 2GM, 76 (33%) OAMFs and 151 (66%) NMFs were found.

Discussion

In this study, strict precautions were taken against possible biases. Bias may arise from unsatisfactory or inconsistent technical quality of the slides and, therefore, new sections were cut from all of the tissue specimens and stained at one laboratory.

All of the slides were re-examined. In the series concerning MIC, only specimens with distinct microinvasion were included. As a consequence, 13 (28%) out of the 47 collected specimens with this diagnosis were excluded. In the study by Sedlis and coworkers, 37% (99 out of 265) of the specimens initially submitted by the participating pathologists in the Gynecologic Oncology Group as MIC were judged to be noninvasive neoplasia[13]. In our MIC series, the surface epithelium associated with the microinvasive area could not be classified as CIN in one specimen. In the study by Ng and coworkers, 1 out of the 66 MICs originated from the base of normal-looking epithelium[9].

The analysis of the mitotic figures was carried out by one person. To minimize intraindividual variation, only the welldefined and clearly recognizable mitotic figures were considered and the rules for mitosis counting were strictly adhered to[15].

The proliferation rate of a lesion is commonly expressed as the number of mitoses per 10 high power fields, but this method has the disadvantage that the outcome depends on the cell size and the square area of the high power field[4]. An additional problem is

met in the study of MIC, as invasion often begins as tiny islands which are not large enough to fill a high power field. Therefore, we assessed the MI, i.e. the number of mitoses per 1000 nuclei. For the assessment of the MI, the first series of slides were used.

We found that intraepithelial part of MIC is characterized by a minimum MI value of 3 and the presence of 2GM among 228 classified mitoses. The findings regarding the MI and the various mitotic figures in the CIN areas of the specimens with MIC can not be compared directly to the previously reported findings in the CIN III lesions without concomitant invasive growth[16]. The estimates of the MI in the intraepithelial area of specimens with MIC are conservative. The MIC specimens were collected from the pathology laboratories of 10 different hospitals and only part of the cervical transformation zone was available for study in many cases. In our previous study on CIN, the whole transformation zone could be studied and the results were based on the area with the highest MI in the whole lesion.

No difference was found between the intraepithelial and invasive area of MIC with regard to the distribution of the MI. However, the lag type mitoses, in particular the 2GM, occurred significantly less often in the invasive area than in the intraepithelial area. We suggest that the lower frequency of lag type mitoses in the invasive area relates to the common finding that the cells in the microinvasive foci are better differentiated than those in the associated CIN[6]. A high frequency of lag type mitoses might signify great genetic instability. As compared to the intraepithelial part, the microinvasive focus might constitute a genetically more stable cell population, possibly resulting from clonal selection. No other reports were found regarding the quantitative occurrence of lag type mitoses in different areas of specimens with MIC.

We studied two groups of patients without cervical neoplasia. We found that the MI ranged from 0 to 4 with a median value of 1 in the non-neoplastic cervical changes of 31 women with reported cytological abnormality. In the non-neoplastic cervical changes of 51 patients whose uterus was excised for non-cervical uterine disorders, the MI ranged from 0 to 5 with a median value of 1. The findings regarding the MI were similar in both groups. Very few other reports have appeared on the analysis of mitoses in non-dysplastic cervical epithelium. Chi et al reported a mean MI of 1.8 in normal cervical

epithelium, but no range of values was given 2. No mitoses in normal epithelium were found by another group of investigators[14].

As regards the mitotic figures in non-neoplastic lesions, no other reports were found that could be used for a comparison with our findings. The few reports on mitotic figures in non-neoplastic changes are concerned with particular histological entities, such as flat condylomata or immature metaplasia[3,17]. In the present study, the findings regarding the mitotic figures were similar in both groups. All of the proliferative (MI ≥2) benign changes in both groups showed a high percentage of so called 'other atypical mitotic figures'. Normal mitotic figures may look asymmetrically after being sectioned slantwise. Hence, these figures were scored as OAMFs because we strictly adhered to the definitions for the mitotic figures. No 3GM could be demonstrated in either group with non-neoplastic changes. The presence of 2GM among 228 classified mitoses could be demonstrated in 5 out of the 82 patients of both groups with non-neoplastic changes. In all of the 5 cases, the slides were of good technical quality and no severe inflammation interfered with the examination. Four out of the 5 specimens showed mature metaplasia with a mitotic index of 2. We do not know whether the presence of an occasional 2GM in mature metaplasia implicates a propensity for future neoplasia. The fifth case concerned basal cell hyperplasia with a MI of 3 and 3 2GMs among 228 classified mitoses. In this very same report, we have concluded that the intraepithelial part of the MIC lesion is characterized by a minimum MI value of 3 and the presence of 2GM among 228 classified mitoses. The findings in our patient with basal cell hyperplasia are scientifically intriguing. Basal cell hyperplasia has been considered a precursor of carcinoma[5], and we are thus confronted with a diagnostic problem concerning the morphological definition of precursors of cervical carcinoma.

In conclusion, we have demonstrated that the intraepithelial area in MIC lesions is characterized by a minimum MI value of 3 and the presence of 2GM among 228 mitoses. This combination of features does not occur in non-neoplastic epithelium, apart from an exceptional case of basal cell hyperplasia. LTMs indicate malignancy if they can be found easily. LTMs can also be found occasionally in non-neoplastic epithelium and, therefore, their presence is not pathognomonic of malignancy. Mitotic figures other than LTMs do not seem to have any diagnostic importance.

Acknowledgement

The authors gratefully acknowledge the contributions made to the study by the pathologists from the laboratories in Assen, Groningen, Hoogeveen, Leeuwarden and Winschoten.

References

1. Anderson M C (1985) The pathology of cervical cancer. Clin Obstet Gynaecol 12:87-119
2. Chi CH, Rubio CA, Lagerlof B (1977) The frequency and distribution of mitotic figures in dysplasia and carcinoma in situ. Cancer 39:1218-1223
3. Crum CP, Egawa K, Fu YS, Lancaster WD, Barron B, Levine RU, Fenoglio CM, Richart R M (1983) Atypical immature metaplasia (AIM). A subset of human papilloma virus infection of the cervix. Cancer 51:2214-2219
4. Ellis PSJ, Whitehead R (1981) Mitosis counting. A need for reappraisal. Human Pathol 12:3-4
5. Farber E, Cameron R (1980) The sequential analysis of cancer development. Adv Cancer Res 31:125-226
6. Ferenczy A, Winkler B (1987) Carcinoma and metastatic tumors of the cervix. In: Kurman RJ (ed) Blaustein's pathology of the female genital tract, 3rd ed. Springer, New York, p 221
7. Ingelfinger JA, Mosteller F, Thibodeau LA, Ware JH (1983) Biostatistics in clinical medicine. Macmillan, New York, pp 169-170
8. Mourits MJE, Pieters WJLM, Hollema H, Burger MPM (1992) Three group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 167:591-595
9. Ng ABP, Reagan JW (1969) Microinvasive carcinoma of the uterine cervix. Am J Clin Pathol 52:511-529
10. Pieters WJLM, Koudstaal J, Ploem-Zaaijer JJ, Janssens J, Oosterhuis JW (1992) The three group metaphase is a morphological indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 14:227-232.
11. Richart RM (1973) Cervical intraepithelial neoplasia: a review. In: Sommers SC (ed) Pathology Annual. Appleton-Century-Crofts, New York, pp 301-328
12. Rubio CA. (1991) Atypical mitosis in colorectal adenomas. Path Res Pract 187:508-513
13. Sedlis A, Sall S, Tsukada Y, Park R, Mangan C, Shingleton H, Blessing JA (1979) Microinvasive carcinoma of the uterine cervix: a clinical-pathologic study. Am J Obstet Gynecol 133:64-74
14. Tanaka T (1986) Proliferative activity in dysplasia, carcinoma in situ and microinvasive carcinoma of the cervix. Path Res Pract 181:531-539
15. Van Diest PJ, Baak JPA, Matze-Cok P, Wisse-Brekelmans ECM, Van Galen CM, Kurver PHJ, Bellot SM, Fijnheer J, Van Gorp LHM, Kwee WS, Los J, Peterse JL, Ruitenberg HM, Schapers RFM, Schipper MEI, Somsen JG, Willig AWPM, Ariens ATh (1992) Reproducibility of mitoses counting in 2,469 breast cancer specimens. Results from the multicenter morphometric mammary carcinoma project. Human Pathol 23:603-607
16. Van Leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and mitotic index in cervical intraepithelial neoplasia. Virchows Archiv 1995 (in press).
17. Winkler B, Crum C, Fujii T, Ferenczy A, Boon M, Braun L, Lancaster WD, Richart RM (1984) Koilocytotic lesions of the cervix. The relationship of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 53:1081-1087

CHAPTER 4

THE COMPARISON OF TWO DIAGNOSTIC MODELS FOR CERVICAL NEOPLASIA

Summary

Objective: To analyse the suitability of the quantitative classification model for diagnosing cervical neoplasia and compare it to the conventional qualitative classification model. The quantitative model is based on the analysis of mitoses: according to this model, a major intraepithelial lesion shows more than 3 mitoses per 1000 nuclei and two-group metaphases among 228 mitoses. The qualitative model is the conventional classification system for cervical neoplasia with three grades of intraepithelial lesions.
Design: Survey.
Setting: Gynecological outpatients clinic of the University Hospital.
Subjects: 225 women with abnormal cytology reports and CIN in the colposcopically directed biopsies.
Mean outcome measures: 1) Number of CIN lesions which could not be classified with the qualitative model; 2) discrepancies between the biopsy diagnosis and the final diagnosis according to the quantitative and qualitative models. The final diagnosis was the most severe lesion on any of the patient's specimens.
Results: ad 1) In the biopsies from 17 (8%) out of the 225 women, the lesion was too small or contained too few mitotic figures to enable the classification of 228 mitoses. In the tissue specimens obtained from the excised transformation zone from 9 (4%) out of the 225 women, the lesion did not enable the counting of 228 mitoses. These 26 cases were excluded from the analysis.
ad 2) With the quantitative model, the biopsy diagnosis underestimated the final diagnosis in 35 (18%) out of the remaining 199 cases. With the qualitative model (classes CIN I, CIN II-III and carcinoma), The biopsy diagnosis underestimated the final diagnosis in 12 (6%) out of the 199 patients.
ad 3) A major intraepithelial lesion according to the quantititive model was present in 13 (48%) out of the 27 patients with CIN I, in 20 (57%) out of the 35 patients with CIN II, and in 122 (90%) out of the 135 patients with CIN III.
Conclusions: ad 1) If the lesions are small or mitoses are scarce, it may be impossible to make a diagnosis with the quantitative model.
ad 2) There was a tendency towards fewer discrepancies between the biopsy diagnosis and the final diagnosis using the qualitative model.

3) About half of the lower CIN grades were classified as major intraepithelial lesions with the quantitative model; these lesions should be considered for treatment.

Introduction

Cervical intraepithelial neoplasia (CIN) is a morphologically defined lesion associated with the development of invasive carcinoma. CIN is subdivided into three grades according to the degree of cellular atypia and disturbance of the epithelial architecture. In the classical morphogenetic model, the lesion starts with CIN I and progresses through CIN II and CIN III to invasive carcinoma[9]. In practice, the CIN classification is hampered by a substantial inter- and intraobserver variation. It is particularly difficult to distinguish the lower CIN grades from the nondysplastic cervical lesions[5,10]. The variability results from the use of multiple classification criteria that can occur independently and which are not dichotomous, but have to be classified on a continuous scale. In addition, the definitions of the various classification models for intraepithelial processes are not uniform[1]. We refer to the conventional system as the qualitative model for diagnosing cervical neoplasia.

Any alternative classification model designed to avoid the disadvantages of the conventional CIN classification must be based on a limited number of distinct features which are dichotomous (present/absence) or quantifiable. We developed a so-called quantitative model which is based on the mitotic index and presence of 'lag type' mitotic figures. The mitotic index (MI) is defined as the number of mitoses per 1000 nuclei. The lag type mitoses of interest are the two group metaphase (2GM) and the three group metaphase (3GM), which show nonattached condensed chromatin at one or two side(s), respectively. A pivotal element in the development of the quantitative model was the finding that 3GM was present in the intraepithelial part of 93% of the microinvasive carcinomas[6], whereas this figure did not occur in CIN I lesions[12]. In our opinion, the 3GM can be regarded as a marker of CIN and assessment might be particular useful for discriminating benign reactive changes from CIN[12]. A drawback in practical terms is the scarcity of the 3GM in many lesions. In a previous study, we demonstrated that the 3GM is strongly associated with the presence of 2GM and MI[11]. An MI ≥ 3 and the presence of 2GM, which is much easier to detect than the 3GM, can be substituted for the presence of 3GM[11]. On the basis of the results of these previous studies, we defined a major

lesion according to the quantitative model as a lesion which showed 2GM combined with an MI ≥3. A minor change showed an MI ≤2 or no 2GMs among 228 mitoses. By counting 228 mitoses, we were 99% confident that any particular mitotic figure was detected which actually made up at least 2% of the mitoses[11].

To evaluate whether the quantitive model is suitable for practical use and how it compares to the conventional CIN classification, we assessed 1) the number of CIN lesions which could not be classified using the quantitative model; 2) discrepancies between the biopsy diagnosis and the final diagnosis within each of the two models, and 3) differences between the final diagnosis according to both models. The study was performed as a cross-sectional study on newly diagnosed patients with CIN.

Materials and methods

Patients

Patients were recruited from the outpatient cervical colposcopy clinic of the Department of Gynecology, University Hospital Groningen.

All of the patients had two consecutive mildly to moderately dyskaryotic cervical smears or one severely dyskaryotic cervical smear. These indications correspond with the grounds for performing colposcopy as agreed by cytopathologists and gynecologists in the Netherlands. In case of mild to moderate dyskaryosis, the maximum interval between two abnormal smears was one year. In addition, CIN had be diagnosed in the colposcopically directed biopsies of all patients. Patients were not included if they had undergone previous colposcopic examination because of an abnormal cytology report indicating intraepithelial neoplasia or their cylindrical epithelium contained atypical cells or they were pregnant. From 1 September 1988 up to 1 January 1994, 261 consecutive patients were eligible for study. 36 Cases were excluded because their specimens did not contain qualitatively satisfactory material for morphological analysis (n=11), or there were no specimens available for examination (n=25). Therefore, this study was performed on the specimens of 225 patients. The age of the patients ranged from 21 to 66 years with a mean of 34.5 (SD 7.4) years.

Tissue processing

If CIN was diagnosed in the biopsies, the whole transformation zone was excised about 6 to 10 weeks later by either loop electrosection (LETZ) or cold-knife conization. Both treatment techniques rendered the entire lesion available for morphological examination. The diathermy loop was used when the squamocolumnar junction could be visualised entirely and did not extend up into the canal for more than 5mm, measured from the anatomical os externum. The tissue was fixed immediately in buffered formalin 8%, Ph 7.42. After paraffin embedding, at least four sections of 4 μm thickness were cut in an anterior-posterior direction and processed routinely for HE staining as described[3].

Cold knife conization was performed when the neosquamocolumnar junction extended up into the endocervical canal for more than 5 mm, measured from the anatomical os externum. For histopathological analysis, the excised cone was incised at the 12 o'clock position, stretched, fixed in formalin and embedded in paraffin. At each hour position, a section of 4 μm thickness was cut and the 12 sections obtained were processed routinely for HE staining.

The histological diagnosis of CIN according to WHO criteria[14] and MIC according to the FIGO rules[4] was independently made by the 2 pathologists (HH and WJLMP). In case of a discrepancy, cases were re-evaluated together and a consensus diagnosis was made. The lesions showing the most severe lesion was taken as the final diagnosis. The pathologists were unaware of the mitoses counting results.

In every patient, we identified the area with the highest cellproliferation in the biopsies and in the electrosectioned or cold knife cone. In these two areas, the MI was assessed by counting the number of mitoses per 1000 nuclei from basal to proximal through the epithelial layer. If the MI ≥3, 228 mitoses were classified. By counting 228 mitoses, we were 99% confident that any particular mitotic figure was detected which actually made up at least 2% of the mitoses[6]. If the area was too small to count 228 mitoses, we used additional odd-numbered step sections.

To read the mitotic figures, we adhered to the recommendations of Van Diest et al[13] who propossed that the nuclear membrane should be absent indicating that the nucleus

had passed the prophase and clear hairy extension of nuclear material should be present. Pycnotic nuclei with basophilic cytoplasm were not considered.

The 2GMs and 3GMs were defined as atypical mitotic figures which showed nonattached condensed chromatin at one of both polar sides, respectively.

Major intraepithelial lesions were defined as lesions which showed an MI≥3 with 2GM or 3GM among 228 mitoses, whereas minor changes were defined as intraepithelial lesions without these features.

Statistical analysis.

The data were analysed using the SYSTAT software package[14]. To test for a significant difference between two groups regarding a qualitiative or quantitative variable, the chi-square test and the Mann-Whitney U test were used, respectively. P values of ≥0.05 were considered to be significant.

Results

CIN lesions which could not be classified according to the quantitative model.

The biopsies of 17(8%) out of the 225 patients contained lesions which were too small or showed too few mitoses to be enable to classify 228 mitoses. The tissue specimens from excised transformation zones of 9 (4%) out of the 225 did not enable the counting of 228 mitoses for similar reasons. These 26 cases were not included in the further analysis.

Analysis of the quantitative model.

Among the remaining 199 patients, the final diagnosis according to the quantitative model were a minor lesion in 42 (21%), a major lesion in 155 (78%) and microinvasive carcinoma in 2 (1%).

Table I shows the relation between the biopsy diagnosis and the final diagnosis according to the quantitative model. The biopsy diagnosis underestimated the final diagnosis in 36 (18%) out of the 199 patients. The number of punch biopsies taken in these 36 cases ranged from 1 to 6, with a mean of 2.7 (SD 0.9) biopsies. No statistically significant differences was found between the two groups with regard to the number of punch biopsies taken (p=0.57; mann-Whitney U test). In the 34 patients whose biopsy diagnosis was a minor lesions and the final diagnosis was a major intraepithelial lesion, 29 cases showed an MI≤1 in their biopsies, 4 showed an MI value of 2 and 1 patient showed an MI≥3. In both the cases a final diagnosis of microinvasive carinoma, the biopsy diagnosis showed a major intraepithelial lesion.

Table 1. Biopsy diagnosis in relation to the final diagnosis according to the quantitative model.

biopsy diagnosis	final diagnosis		
	minor lesions	major lesions	total
minor lesions	42	34	76
major lesions		121	123
total	42	160	199

Analysis of the qualitative model.

The final diagnosis on the 199 patients were CIN I in 27 (13%), CIN II in 35 (18%), CIN III in 135 (68%) and microinvasive carinoma in 2 (1%). Table 2 shows the relation between the biopsy diagnosis and the final diagnosis. The biopsy diagnosis underestimated the final diagnosis in 35 (18%) out of the 199 patients. In these patients, the number of punch biopsies taken ranged from 1 to 5 with a mean of 2.6 (SD 0.9). No statistically significant difference could be demonstrated between these two groups with regard to the number of punch biopsies taken (p=0.31; Mann-Whitney U test).

blz 70

Table 1

Biopsy diagnosis in relation to the final diagnosis according to the quantitative model.

biopsy diagnosis	final diagnosis			
	minor lesions	major lesions	MIC	totals
minor lesions	42	34		76
major lesions		121	2	123
totals	42	155	2	199

blz 72

Table 3.
Relation to the final diagnoses according to the quantitative and qualitative model diagnostic models.

	Final CIN grade				
	I	II	III	MIC	total
Minor lesion	14	15	13		42
Major lesions	13	20	122		155
Carcinoma				2	2
totals	27	35	135	2	199

ad blz 92

Table II. p53 expression in relation to CIN grade and HPV.

CIN grade	HPV-type				
	II	III	no	oncogenic(♦)	X(*)
P53 expression					
0-1%	6	20	2	19	5
2-5%	3	5	0	6	2
6-10%	2	3	2	3	0
11-25%	0	2	0	2	0
26-50%	1	3	2	2	0
>50%	0	7	1	5	1

♦: oncogenic HPV referers to HPV-16, 18, 31, 33 or a combination of these types. Two cases in combination with HPV 6 or 11.
*: X: HPV positive but not type 16, 18, 31, 33, 6 or 11.

As the qualitative diagnostic model has four categories (CIN I, CIN II, CIN III, and microinvasive carinoma) and the quantitative model has three categories (minor change, major change and microinvasive carcinoma), there is a greater probability on the basis of chance alone that the biopsy diagnosis will underestimate the final diagnosis using the qualitiative model. A more valid comparison was possible after we combined two CIN categories. When we dichtomized CIN at the level between CIN II and CIN II (i.e. the classes were CINI-II, CIN III and carcinoma), the biopsy diagnosis underestimated the final diagnosis in 31 (16%) out of the 199 patients. This level of underestimation was similar to that obtained with the quantitative model (31/199 versus 36/199; p=0.45 from chi-square test). However, when we dichotomized CIN at the level between CIN I and CIN II (i.e. the classes were CIN I, CIN II-III and carcinoma), the biopsy diagnosis underestimated the final diagnosis in 12 (6%) out of the 199 patients. Using these categories, the level of underestimation was significantly lower with the qualitative model (12/199 versus 36/199; p<0.001 from chi-square test). We conclude that there were fewer discrepancies between the biopsy diagnosis and the final diagnosis using the qualitative model.

Table 2. Biopsy diagnosis in relation to the final diagnosis according to the qualitative CIN model.

biopsy diagnosis	final diagnosis				
	CIN I	CIN II	CINIII	MIC	total
CIN I	27	4	6		37
CIN II		31	23		54
CIN III			106	2	108
total	27	36	138	2	199

Comparison of the final diagnosis.

Table 3 shows the final diagnoses according to the two diagnostic models. We found a major intraepithelial lesions according to the quantitative model in 13 (48%) out of the

27 patients with CIN I, in 20 (57%) out of the 35 with CIN II, and in 122 (90%) out of the 135 with CIN III. Thirteen patients with CIN III had a minor change. Nine out of these 13 patients had a lesion which showed and MI≤2 and in the reamining 4 patients, the 2GM was not found among the 228 mitoses.

Table 3. Relation to the final diagnoses according to the quantitative and qualitative model diagnostic models.

	Final CIN grade				
	I	II	III	MIC	total
Minor lesion	14	15	13		45
Major lesions	13	20	122		155
Carcinoma				2	
total	27	35	135	2	199

Discussion

We took many precautions to obtain valid results. The patients comprised a large group of consecutively enrolled women who showed at least CIN I in their biopsies. Patients with an abnormal cytology report but without CIN in their biopsies were excluded because we could not justify excising their transformation zone. The colposcopic examinations were performed by three experienced oncological gynecologists, in order to ensure adequate tissue sampling. The tissue sections were cut and stained at one laboratory (Laboratory of Pathology, Winschoten); this condition produced slides of constant quality. CIN was graded indepently by the two pathologists (HH and WJLMP) and a concensus diagnosis was made if there were discrepancies. The MI was based on the number of mitoses among 1000 nuclei; we have previously demonstrated that examining 10,000 nuclei did not produce a different estimate of the relative number of mitoses[11]. One of the investigators (AMvL) analysed 228 mitoses according to the rules for the analysis of mitoses and technically unsatifactory specimens were excluded from the

analysis[2]. There is less inter-and intraobserver variability in the assessment of the 3GM than for grading CIN[7]. On theoretical grounds there is less observer variability using the quantitative model than the qualitative model. The quantitative model considers only two characteristics which are clearly recognizable and quantifiable. The qualitative model is based on a variety of characteristics which can occur independently and have to be estimated on a scale.

The results of the present study show that the quantitative model has two disadvantages compared to the qualitative model. At the first place, examination according to the quantitative model requires larger sections or additional step sections. In 17 (8%) out of the 225 patients in the present study, the biopsy areas were too small or showed too few mitoses to enable the classification of 228 mitoses. In the second place, a discrepancy tended to occur more frequently between the biopsy diagnosis and the final diagnosis using the quantitative model. The quantitative model is based on only two characteristics (the MI and the presence of 2GM or 3GM). In contrast, the qualitative model incorporates a great diversity of changes and therefore, is defined more comprehensively. We suggest that the comprehensive definition of the qualitative model led to fewer discrepancies between the biopsy diagnosis and the final diagnosis, particularly when the spectrum of changes was dichotomized at the level between CIN I and CIN II.

The present study also showed that the quantitative model may contribute to the better identification of cervical lesions which should be treated or left untreated. The more comprehensive definition of the qualitative model complicates the proces of discriminating between the lower CIN grades and benign reactive changes[5]. In the present study, we showed that 33 (53%) out of the 62 CIN I-II lesions were diagnosed as major intraepithelial lesions according to the quantitative model. We have reported previously that all of the intraepithelial lesions associated with microinvasive carcinoma were major lesions according to the quantitative model. In contrast and apart from a exceptional case, all of the non-neoplastic lesions in women with cytological abnormalities were diagnosed as minor changes[12]. The analysis of mitotic figures, therefore, seems to be helpful for discrimating neoplasia from benign changes. Although there is some doubt about the need of treating CIN I or CIN II, we suggest that the lower CIN grades, which are classified as major lesions according to the quantitative model should be considered for treatment.

References

1. Anderson MC, Brown CL, Buckly CH, Fox H, Jenkins D, Lowe DG, Manners BTB, Melchers DH, Robertson AJ, Wells M. Current views on cervical intraepithelial neoplasia. J Clin Pathol 1991;44:969-978.
2. Baak JPA. Mitosis counting in tumors. Editorial. Hum Path 1990;21:683-685
3. Burger MPM, Hollema H. The reliability of the histological diagnosis in colposcopically directed biopsies. A plea for LETZ. Int J Gynecol Pathol 1993;3:385-90.
4. FIGO Cancer committee. Staging Announcement. Gynecol Oncol 1986; 25:181-5.
5. Ismail SM, Colcough AB, Dinnen JS, et al. Observer variation in histopathological diagnosis and grading of cervical intraepithelial neoplasia. Br Med J 1989;298:707-10.
6. Mourits MJE, Pieters WJLM, Hollema H, Burger MPM. Three group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 1992;167:59-5.
7. Pieters WJLM. Atypical mitotic figures as markers in the classification of squamous cell lesions of the uterine cervix. An evaluation of morphological criteria. Thesis University of Groningen 1987 (in Dutch).
8. Poulsen HE, Taylor CW, Sobin LH. Histological typing of female genital tract tumours. World Health Organization, Geneva 1975.
9. Richart RM. Cervical intraepithelial neoplasia: a review. In: Sommers SC, ed. Patholofy annula, 1973. New York: Appleton-Century-Crofts, 1973:301-28.
10. Robertson AJ, Anderson JM, Swanson Beck J, et al. Observer variability in histopathological reporting of cervical biopsy specimens. J Clin Pathol 1989;42:231-8.
11. Van Leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and Mitotic Index in Cervical Intraepithelial Neoplasia. Virchow Arch 1995;427:139-44.
12. Van Leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and Mitotic Index in Microinvasive carcinoma and in non-dysplastic changes of the uterine cervix. J Obstet Gynecol 1996;16:269- 76.
13. Van Diest PJ, Baak JPA, Matze-Cok P, Wisse-Brekelmans ECM, Galen CM van, Kurver PHJ, Bellot SM, Fijnheer J, Gorp LHM van, Kwee WS, Los J, Peterse JL, Ruitenberg HM, Schapers RFM, Schippers MEI, Somsen JG, Willig AWPM, Ariens Th. Reproducibility of mitoses counting in 2,469 breast cancer specimens. Results from a multicenter morphometric mammary carcinoma project. Hum Pathol 1992;23:603-607.
14. Wilkinson, Leland. SYSTAT: The system for Statistics. Evanston, IL:SYSTAT, Inc, 1990.

CHAPTER 5

HUMAN PAPILLOMAVIRUS TYPE INFLUENCES THE EXTENT OF CHROMOSOMAL LAG DURING MITOSES IN CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III

Summary

The risk of malignant development in the uterine cervix depends on the type of human papillomavirus (HPV) present. We examined whether the HPV type influences the proliferation rate and occurence of mitotic figures with lagging chromosomes in the precursor of cervical carcinoma.

The study group comprised 180 women who were referred because of cytological changes indicating dysplasia and who were subsequently diagnosed with cervical intraepithelial neoplasia grade III.

The HPV-16 associated lesions showed a significantly higher number of mitoses per 1000 nuclei than the lesions without HPV ($p<0.001$). The HPV-16 associated lesions also showed a significantly higher proportion of mitotic figures with lagging chromosomes than the HPV-18 or HPV-31 associated lesions and lesions wihtout HPV ($p=0.01$, $p=0.007$ and $p=0.002$, respectively).

Our results indicate that the differences in oncogenic potential among HPV become apperent in the precursor lesions through the differences in the extent of chromososmal lag during mitoses.

Introduction

Cervical intraepithelial neoplasia (CIN) is morphologically defined lesion associated with the development of cervical carcinoma. CIN is separated into three grades according to the degree of cellular atypia and disturbance of the epithelial architecture. In the classical morphogenetic model, the lesions begins with CIN I and progresses through CIN II and CIN III to invasive carcinoma[14]. However, the scientific pathological basis of the concept has recently been challenged[9]. Knowledge about the involvement of human papillomavirus (HPV) types in the etiology of cervical neoplasia is poorly compatible with gradual progression. The severity of CIN seems to be primarly determinated by the associated HPV type and the topographical position of the lesions on the cervix[2,10,11,15]. In women with cytological changes indicating dysplasia, HPV types 16, 31 and 33 are predominatly found in CIN III[5]. However, comparison of the strength of the associations between the specific HPV types and the different disease severities has indicated that HPV-31 and HPV-33 have less malignant potential than HPV-16[10].

We have recently demonstrated that severity of CIN coincides with increased proliferation rate and increased number of mitotic figures with lagging chromosomes [22]. Lag type mitoses (LTMs) are defined as metaphases with nonattached condensed chromatin in the area of the mitotic figure. Distinctive appearances of the LTMs are two group metaphases (2GM) and three group metaphases (3GM). There are reasonable arguments in support of the view that LTMS signify aneuploidy[22]. Proliferation and chromosomal loss are known to be key features in malignant development[1,17]. As the various HPV types carry different risk for malignant development, we examined whether the HPV type influences the prolferation rate and the occurence of LTMs in CIN III.

Materials and methods

Patients

We recruited patients from the colposcopy clinic of the Department of Gynecology, University Hospital Groningen. The patients had either two cervical smears with changes indicating mild or moderate dysplasia, or one smear indicating severe dysplasia or carcinoma in situ. These cytological abnormalities form grounds for performing colposcopy as agreed by cytopathologists and gynecologists in the Netherlands. At the first visit, we took a cervical scrape for HPV analysis. Four weeks later, we took colposcopically directed biopsies. If CIN was diagnosed from the biopsies, we excised the whole transformation zone by loop electrosection or cold knife conisation. Diathermic loop excision was used if the squamocolumnar junction could be visualized entirely and did not extend up into the canal for more than 5mm from the anatomical os externum. Details of the electrosurgical technique have been described elsewhere[4]. Cervical neoplasia was diagnosed and graded according to the World Health Organization[13]. Patients entered the study group if the most severe lesion was classified as CIN III. Patients were not included if 1) we suspected invasive carcinoma because of clinical signs and/or symptomes; or 2) they had previously undergone colposcopic examination because of an abnormal cytology report; or 3) their cervical smear was taken during pregnancy; or 4) the cervical smear showed atypia of the endocervical glandular epithelium. From 1 September 1988 to 1 September 1993, 180 consecutive patients entered the study group.

Microbiological analysis

The cervix was scraped with the blunt and pointed ends of a wooden cervical spatula and with an endocervical brush. The scraped cells were suspended in 5ml phosphate buffered saline, pH 7.2, supplemented by methiolate 1:10,000 v/v. The samples were analysed for the presence of HPV with the use of a general primer-mediated polymerase chain reaction, as described previously[19]. Positive samples were examined with the polymerase chain reaction for the presence of HPV types 6, 11, 16, 18, 31 and 33 separately, using type specific primers as described previously[12]. If none of these specific types could be detected, the type remained unknown and was designated 'HPV-X". The laboratory staff were unaware of the histological reports.

Morphological examination

For the examination of mitoses, new sections of 4µm thickness were cut from all the tissue blocks, in order to achieve study materal of uniform quality. These sections were used to identify the area with the highest mitotic index (MI). This area could be localized in one of biopsies or in the exconization specimen. The MI was assessed by counting the number of mitoses per 1000 nuclei from basal to proximal through the epithelial layer by using a grid.

Mitotic figures, were defined as figures without a nuclear membrane, which indicated that the cell had passed the prophase, and clear hairy extension of nuclear material should be present. Pycnotic nuclei with basophilic cytoplasm were ignored[21].

Lag type mitoses were defined as mitotic figures with nonattached condensed chromatin in the area of the mitotic figure. LTMs were classified into the following types: two group metaphase (metaphases with nonattached condensed chromatine at one polar side), three group metaphase (metaphase with nonattached condensed chromatine at two polar sides) and other lag type mitoses (LTMs without the configuration of 2GMs or 3GMs). For the purpose of the study, we enumerated the LTMs irrespectively of their subtypes and we did not classify atypical mitotic figures others than LTMs. On arbitrary grounds we wanted to be 99% confident that any particular mitotic figure was detected which actually made up at least 2% of the mitoses. We therefore counted 228 mitoses[22]. If the selected area in the first section (which was also used for the assessment of

the MI) was too small to count 228 mitoses, we used additional odd-numbered step sections. If the MI was 1 or less, the mitotic figures were not analysed in the respective section because it was practically impossible to count 228 mitoses.

The microscopical examinations were performed with a Leitz dialux 20 EB microscope with a 40x npl fluotar Leitz objective (n.a. 0.70) and a 10x wide field periplan ocular piece.

Statistical analysis

To test for a significant difference between groups with regard to a qualitative variable, we used the chi-square test. To test for a significant difference between two groups with regard to a quantitative variable, we used the Mann-Whitney U-test. In the latter analysis, P values of ≤ 0.01 were considered to be significant. The value 0.01, instead of the more commonly used 0.05, resulted from the correction according to Bonferroni which should be applied in case of multiple comparisons[6].

Results

The patients group comprised 180 women with CIN III. The age of the patients ranged from 20 to 66 with a mean value of 34.8 (SD 7.1) years. A total of 11 women harboured multiple HPV types and these women were not included in the analysis.

Table 1 shows the distribution of the cytological diagnosis per HPV type. No association was found between the cytological diagnosis and the HPV type present ($X^2=0.19$, df=5, p=0.99; X^2 tests). No women with HPV-6 or HPV-11 only were present in the patients group.

The MI could not be assessed in 9 patients because of morphological unsatifactory material or insufficient amount of material, which left 160 patients. No preponderance of missing values was seen in particular groups according to HPV type. Figure 1 shows a graphical representation of the distribution of the MI per HPV type and Table I shows the statistical descriptors. The HPV-16 associated lesions showed a significant higher MI value than the lesions without HPV ($p<0.001$; Mann-whitney U test). The HPV-16 associated lesions did not differ from the HPV-18, HPV-31 and HPV-33 associated lesions with regard to the MI value (p=0.08, p=0.04, p=0.29, respectively Mann-whitney U test).

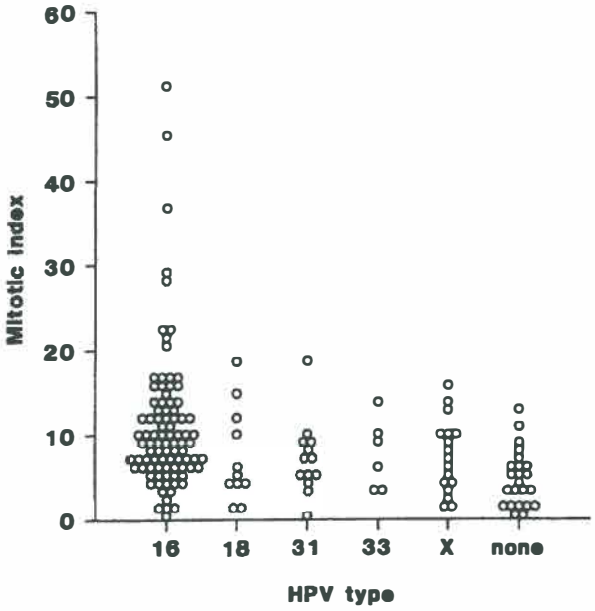


Figure 1: Distribution of the mitotic index per human papillomavirus (HPV) type.

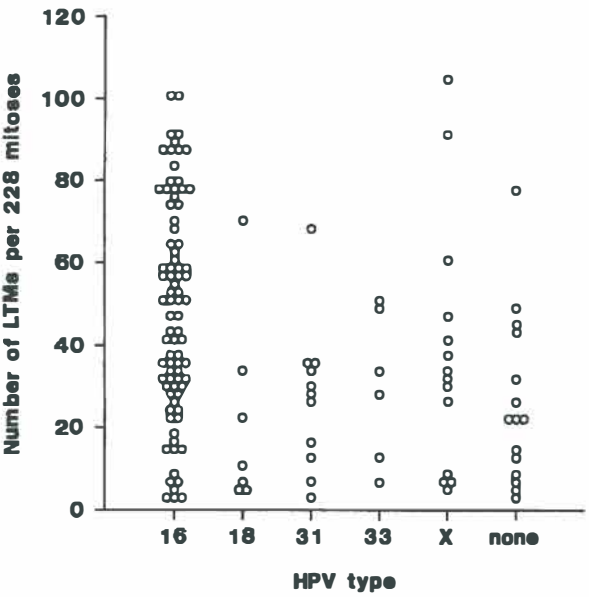


Figure 2: Distribution of the number of lag type mitoses (LTMS) per human papillomavirus (HPV) type.

Table 1. Distribution of selected factors in women with CIN III according to the presence of specific HPV types.

Factor	no HPV	HPV-16	HPV-18	HPV-31	HPV-33	HPV-X
• number of patients	26	93	11	13	8	18
• cytological diagnosis						
- mild to moderate	7	24	3	3	2	4
- severe or CIS	19	69	8	10	6	14
• Mitotic index						
- median vlaue	4	9	5	7	7.5	7
- interquartile limits	1-6	6-13	4-11	5-9	3-10	4-10
• Number of lag type mitoses						
- median	23	48.5	10	27	31	32.5
- interquartile limits	10-38	30-70	5.5-28	15-35	13-48	8-47

The number of LTMs could not be assessed in an additional 25 patients, which left 135 patients. The MI was too low (i.e. 0 or 1) to count 228 mitoses in 16 cases, and the lesion was too small to count 228 mitoses in 9 cases. We found a preponderance of low MI values in the lesions without HPV; the other causes for drop-outs were not associated with any HPV type. Figure 2 shows a graphically representation of the distribution of the number of LTMs per HPV type and Table 1 shows the statistical descriptors. The HPV-16 associated lesions showed a significantly higher number of LTMs than the lesions without HPV or HPV-18 and HPV-31 associated lesions ($p=0.002$, $p=0.01$ and $p=0.007$, respectively Mann-whitney U tests). The HPV-16 associated lesions did not differ from the HPV-33 associated lesions with regard to the number of LTMs among 228 mitoses ($p=0.08$, respectively Mann-whitney U test).

Discussion

We performed this study on a series of consecutively enrolled women who were referred because of cytological changes indicating dysplasia and who were subsequently diagnosed with CIN III. It may be difficult to discriminate the lower CIN grades from benign reactive changes[7]. To avoid including women whose lesion was actually a benign

reactive change, we only studied women with CIN III. Although entry depended on two different levels of the cytological abnormality (either one smear indicating severe dysplasia or carcinoma in situ), there was no association between these categories and the HPV type.

In a previous report we demonstrated that an increased MI coincided with an increased number of LTMs in CIN lesions[22]. In the present study, we found the highest median MI value and the highest median number of LTMs in the HPV-16 associated lesions. The HPV-16 associated lesions showed a significant higher number of LTMs than the HPV-31 associated lesions and the observations with regard to the MI showed a similar trend (p=0.04). We found no difference between the HPV-16 and HPV-33 associated lesions with regard to the MI and the relative number of LTMs, but the number of HPV-33 associated lesions was small. Lorincz et al[10] compared the strength of the association between the specific HPV types and different disease severities. The results indicated that HPV-16 has more malignant potential than HPV-31 or HPV-33. HPV-16 was found with almost equal frequency in the high-grade intraepithelial lesions and invasive carcinomas, which justifies the term 'high risk'. HPV types 31 and 33 were more common in high grade intraepithelial lesions than in invasive carcinomas and were therefore assigned an intermediate-risk status. The different risk status of HPV-16 versus HPV-31 matches our finding that these virus types are associated with different proportions of LTMs and also tend to be associated (p=0.04) with different proliferation rates.

HPV types 16, 31 and 33 show close similarity of the E6 gene: these virusses belong to the same main branch of the phylogenetic tree which was constructed on the basis of the nucleotide and amino acid sequence alignment of the E6 gene[23]. The interaction between the E6 protein from the high-risk HPV types and the p53 gene product results in a rapid degradation of the latter[16]. The wild type p53 protein induces cell cylce arrest in the G1 phase to allow time for the repair of any DNA damage before replication proceeds[8]. This effect of the high-risk HPV E6 gene products may well be linked to the malignant derailment because the E6 protein from the low-risk HPV types 6 and 11 do not complex with the p53 gene product[18,24]. It has been suggested that the interaction between high-risk HPV E6 protein and the p53 protein contributes to cell proliferation[24]. However, the low-risk HPV types 6 and 11 also stimulates host cell proliferation (resulting in condylomata accuminata) although their E6 protein is unable to degrade the p53

gene product. This observation indicates that HPV has an indepent proliferation inducing capacity. Differences in the oncogenic potential of high risk HPV types might be determinate, at least in part, by differences in their proliferation-inducing capacity.

In our series, HPV-18 associated lesions showed comparatively low MI and low proportions of LTMs. However, HPV-18 was assigned a high oncogenic risk status because this virus type was more common in invasive carcinoma than in high-grade CIN. Investigators have suggested that HPV-18 is associated with rapid-transit tumors[10]. The patients in our series had CIN III which is a squamocellular disorder, while HPV-18 is particularly associated with adenocarcinoma and small cell cervical carcinoma[20]. Little is known about the precursors of small cell carcinoma. The existence of precancerous lesions for adenocarcinoma is controversial, because there are no accepted dysplastic conditions of the endocervical gland. Moreover, adenocarcinoma is more difficult to detect in cervical smears than squamous carinoma[3]. Therefore, HPV-18 associated CIN III lesions in women with abnormal smears form a highly selected (biassed) sample of the HPV-18 associated neoplasias. Inferences on the oncogenic potential of HPV-18 do not seem to be justified on the basis of its occurrence in CIN.

References

1. Baak JPA. Mitosis counting in tumors. Hum Pathol 1990;21:683-4.
2. Bergeron C, Barasso R, Beaudenon S, Flamant P, Croissant O, Orth G. Human papilloma viruses associated with cervical intraepithial neoplasia. Great diversity and distinct distribution in low-and high-grade lesions. Am J Surg Path 1992;16:641-9
3. Brand E, Berek JS, Hacker NF. Controversies in the managment of cervical adenocarcinoma. Obstet Gynecol 1988;71:261-9.
4. Burger MPM, Hollema H. The reliability of the histological diagnosis in colposcopically directed biopsies. A Plea for LETZ. Int J Gynecol Cancer 1993;3:385-90.
5. Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predicitve value of human papillomavirus type for histological diagnosis of women with cervical cytological abnormalities. BMJ 1995;310:94-5.
6. Ingelfinger JA, Mosteller F, Thibodeau LA, Ware JH. Biostatistics in clinical medicine. New York: Macmillan 1983;169-70.
7. Ismail SM, Colclough AB, Dinnen JS, Eakins D, Evans DMD, Gradwell E, O'Sullivan JP, Summerell JM, Newcombe RG. Observer variation in histopathological diagnosis and grading of cervical intraepithelial neoplasia. BMJ 1989;298:707-10.
8. Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Hau SM, Lorincz AT, Hedrick L, Vho KR. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc Natl Acad Sci USA 1993;90:3988-92.
9. Kiviat NB, Critchlow CW, Kurman RJ. Reassessment of the morphological continuum of cervical intraepithelial lesions: does it reflect different stages in the progression to cervical carcinoma. In: The epidemiology of cervical cancer and Human papillomavirus. Munoz E, Bosch FX, Shah KV, Meheus A (eds). International Agency for Research on Cancer, Lyon. p59-66.
10. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet Gynecol 1992;79:328-37.
11. Matsukura T, Sugase M. Identification of genital human papilloma virus in cervical biopsy specimens: segregation of specific virus types in specific clinical pathologic lesions. Int J Cancer 1995;61:13-22.
12. Melchers W, Vanden Brule A, Walboomers J, De Bruin M, Burger M, Herbrink P, Meijer C, Lindeman J, Quint W. Increased detection rate of human papillomavirus in cervical scrapes by polymerase chain reaction as compared to modified FISH and Southn-blot analysis. J Med Virol 1989;27;329-35.
13. Poulsen HE, Taylor CW, Sobin LH. Histological typing of femal genital tract tumors. World Health Organization, Geneva 1975.
14. Richart RM. Cervical intraepithelial neoplasia: a review. In: Sommers SC, ed. Patholgy annual. New York: Appleton-Century-Crofts, 1973;301-28.
15. Saito K, Saito A, Fu YS, Smotkin D, Gupta J, Shah K. Topographis study if cervical condylomata and intraepithelial neoplasia. Cancer 1987;59:2064-70.
16. Scheffner M, Werness BA, Huibregtse JM, Levine JM, Howley PM. The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. Cell 1990;63:1129-36.
17. Shackney SE, Smith CA, Miller BW, Burholt DR, Murtha K, Giles HR, Ketterer DM, Pollice A. A Model for the genetic evolution of human solid tumors. Cancer Res 1989;49:3344-54.

18. Slebos RJC, Kessis TD, Chen AW, Han SM, Hedrick L, Cho KR. Functional consequences of directed mutations in human papillomavirus E6 proteins: abrogation of p53-mediated cell cycle arrest correlates with p53 binding and degradation in vitro. Virology 1995;208:111-20
19. Snijders PJF, Van Den Brule A, Schrijnemakers H, Snow G, Meijer CJLM, Walboomers JMM. The use of general primers in the PCR permits the detection of a broad spectrum of human papillomavirus infections. J Gen Virol 1990;72:2781-6.
20. Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasia. Hum Pathol 1992;23:117-28.
21. Van Diest PJ, Baak JPA and 18 others. Reproducibility of mitosis counting in 2,469 breast cancer specimens. Results from the multicenter morphometric mammary carcinoma project. Hum Pathol 1992;23:603-7.
22. Van leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and the mitotic index in cervical intraepithelial neoplasia. Virchows Arch 1995;427:139-44.
23. Van Ranst M, Kaplan JB, Burk RD. Phylogenetic classification of human papillomaviruses; correlation with clinical manifestation. J Gen Virol 1992;73:2653-60.
24. Werness BA, levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 1990;248:76-9.

CHAPTER 6

P53 EXPRESSION IS NOT RELATED TO THE EXTEND OF CHROMOSOMAL LAG DURING MITOSES IN CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE II AND III

Introduction

The tumor suppressor gene p53 has a major guarding role in maintaining the integrity of the cellular DNA[13]. P53 upregulation causes G1 arrest, resulting in delayed gene amplification whereby genetic damage can be repaired. Recently, Cross et al demonstrated that p53 acts as a spindle checkpoint in mice fibroblastic celllines whereby P53 mutated cells showed mitotic delay and atypical mitotic figures. P53 mutation resulted in endoduplication with the formation of multiple centrioles[9,13].

P53 can be inactivated by allelic loss and mutation[2] and by interaction of viral oncoproteins, including the E6 protein of oncogenic human papillomaviruses (HPV)[40]. HPV infection is associated with cervical neoplasia and may have a keyrole in its development[4]. Cervical carcinoma is preceded by precursor lesions, the cervical intraepithelial neoplasia (CIN). CIN is graded into CIN I, CIN II and CIN III histologically paralleled by increasing nuclear atypia, increasing disturbance or the cellular differentiation and epithelial architecture[39]. CIN lesions harbour HPV in a high percentage of which CIN III showed 80% HPV positivity[6].

High grade CIN demonstrates a high mitotic index and high number of mitoses with lagging chromosomes[45]. The etiology of lag type mitoses is not fully understood but these mitoses may arise because of spindle malformation or abnormal distribution of highploid DNA[35,37,45]. P53 may play a role in the development of lag type mitoses. To study this, we collected a series of women with CIN II or CIN III in which p53 protein expression was related to the number of lag type mitoses.

Materials and methods

Patients were recruited from the colposcopy clinic of the Department of Gynecology of the University Hospital Groningen. They were referred because of abnormal cervical cytology indicating dyskaryosis. They were eligible for participation in the study if they had abnormal cervical cytology indicating CIN and subsequently had cervical histology indicating CIN II or CIN III. From 1 January 1991 to 1 January 1994, 74 patients fitted the criteria to enter the study. Out of the 74 patients, 22 were excluded; 10 because of morphologically unsatisfactory material to score the immunohistochemical staining and

10 cases because their CIN lesions were not large enough to count 228 mitoses, and 2 cervical scrapes revealed insufficient material for PCR analysis. Out of the 52 cases, CIN III was found in 40(77%) and CIN II in 12 (23%) patients.

At least 6 weeks before colposcopical examination the cervix was scraped to obtain material for HPV analysis. During colposcopy, biopsies were taken and if the histology indicated CIN I or more the whole transformation zone was excised either by cold knife conization or by diathermic loop excision[6].

Mitotic figures were analysed as described[45]. In short, from the whole transformation zone (including the biopsy) the CIN lesion with the highest number of mitoses per 1000 nuclei mitoses was indentified. In this area, we calculated 228 mitoses to be 99% confident that one lag type mitoses was detected given a frequency of 2%. Lag type mitoses were defined as mitotic figures with nonattached condensed chromatin in the area of the mitotic figure. We considered the two group metaphase (2GM) and the three group metaphase (3GM) which were defined as metaphases with nonattached condensed chromatine at one or two polar sides, respectively[45]. The absolute number of 2GM + 3GMs (LTMs) per 228 mitoses was used for analysis.

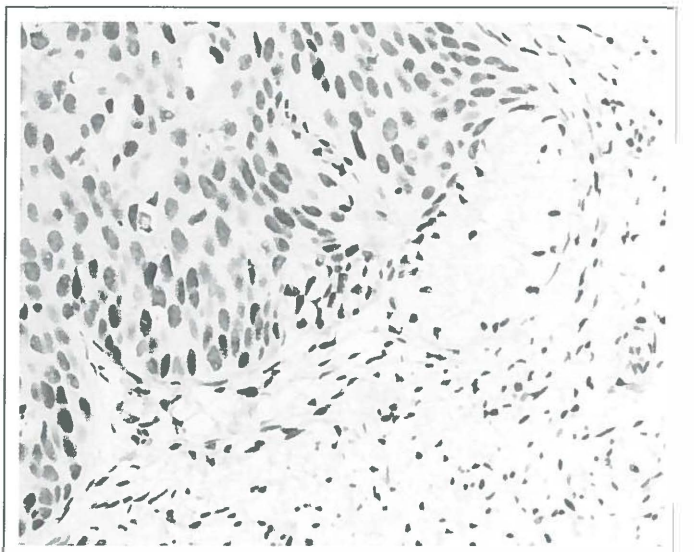
Human papillomavirus type was determined by general primer mediated polymerase chain reaction[42] and type-specific primer mediated polymerase chain reaction for the presence of HPV types 6,11,16,18,31,33 separately[33]. If the general primer was positive but the type-specific PCR was negative, the type remained unknown.

For the detection of p53 expression, we used 4µm sections cut from this tissue blocks which were used for mitotic counting. The sections were mounted on APES coated slides (amino-propyl-ethoxy-silan; SIGMA), deparaffinized, rehydrated to 96% alcohol and air dried. For antigen retrieval, we used an autoclave in which slides were heated 3 times 5 min at 115°C in Blocking reagent (Boehringer Mannheim) (2% block + 0.2% SDS in maleic acid, pH=6.0). After antigen retrieval slides were respectively incubated with a 800x diluted Bp53-12, a monoclonal antibody recognizing wild and mutant protein (BioGenex, San Remon CA). Two step immunostaining was performed according to the manufacturer's procedure of the BioGenex kit, containing anti-mouse biotin and conjugated streptavidin. BCIP-NBT (bromochloroindolyl-phosphate 4-nitro-blue-tetrazoliumchloride; Boehringer Mannheim) was used as a substrate. Sections were counterstained with haematoxilin and mounted with mounting medium. As negative control IgG2a was used instead of p53. P53 expression was semiquantitatively scored

into the following categories: no staining or focally a single positive cell (category 1), 2 to 5 % (2), 6 to 10% (3), 11 to 25% (4), 26 to 50% (5) and more dan 50% positive cells (category 6).

Results

Immunoreactivity for p53 was restricted to the epithelium and localized in the nucleus only. Half (26 out of 52) of the cases expressed ≤1% p53 positive cells. The individual p53 positive cells were generally located in the middle or upper layer of the epithelium. Koilocytes were in general p53 positive. Eight cases demonstrated 2-5% positivity, 2 cases showed 11-25% p53 expression, 4 patients showed 26 to 50% positivity and in 7 cases more than 50% of the cells showed p53 positivity. P53 was not expressed in cells in mitosis (photograph 1).



P53 Immunostaining in CIN III (category 6).
Note negative staining in mitotic figures.

The number of lag type mitoses ranged from 0 to 63 with a median of 10.5. LTMs were present in 9 out 12 (75%) CIN II lesions and in 35 out of 40 (87.5%) CIN III lesions. In CIN II lesions, the median number of LTMs was 2.5 (range 0 to 45) whereas in CIN III the median number of LTMS was 12 (range 0 to 63)

HPV was present in 45(86%) cases. The various types are depicted in Table 1 and lumped into 3 groups. First, HPV negative cases, secondly HPV 16,18,31, 33 or a combination of these types and last a group of unknown HPV types. The relation between p53 expression and HPV types is shown in Table 2. P53 was not expressed in 50% of the cases with oncogenic HPV types. HPV negative cases showed in 7 out of the 9 cases p53 expression which was not a statistically significant finding. Among the unknown HPV types 7 out of 8 cases showed in less then 5% of the cells p53 expression.

Table 1. The distribution of the various HPV types in cervical smears of patients with mild to moderate or severe dyskaryosis.

HPV type	number of patients
no HPV	7
6 or 11, 16	1
6 or 11, 16, 31	1
16	23
16, 18	1
16, 31	2
18	3
31	3
31, 33	2
33	1
unknown type	8
total	52

Figure I demonstrates the relation between the number of lag type mitoses and the p53 expression. No correlation was found between the expression of p53 and the number of lag type mitoses (Spearman rank correlation coefficient r=-0.17).

We studied whether the presence of HPV influenced the relation between p53 and LTM. Therefore, the Spearman rank correlation coefficient between the various p53 classes and LTMs per HPV group was assessed. The Spearman rank coefficients calcu-

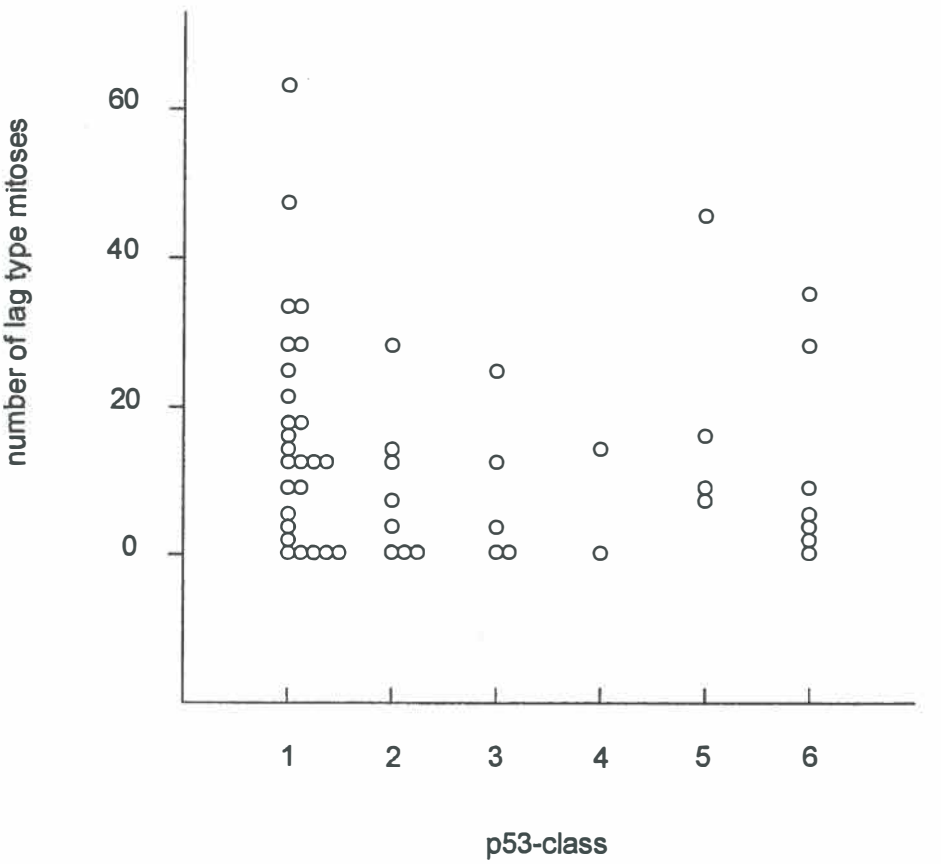
Table 2. p53 expression in relation to CIN grade and HPV.

	CIN grade		HPV-type		
	II	III	no	oncogenic(♦)	X(*)
P53 expression					
0-1%	6	20	2	19	5
2-5%	3	5	0	6	2
6-10%	2	3	2	3	0
11-25%	0	2	2	3	0
26-50%	1	3	2	2	0
>50%	0	7	1	5	1

♦: oncogenic HPV referers to HPV-16, 18, 31, 33 or a combination of these types. Two cases showed a combination of an oncogenic type with HPV 6 or 11.

*: X: HPV positive but not type 16, 18, 31, 33, 6 or 11.

Figure I: The distribution of the number of lag type mitoses in relation tp p53 expression.



lated was 0.20 for the cases without HPV, -0.29 for the cases with oncogenic HPV types and 0.56 for the cases with an unknown HPV type. The correlation between p53 and LTMs in the 8 cases with an unknown HPV type must be coincidental since among the 8 cases, 4 showed neither p53 expression nor LTMs.

Discussion

Previous studies have shown that atypical mitoses with lagging chromosomes are related to high grade CIN[45], and microinvasive carcinomas[35,46]. The etiology of lag type mitoses is still a matter of speculation. It has been suggested that these mitoses might occur because of spindle malformation[17,28,37]. Abnormal distribution of highploid DNA has also been proposed[38]. P53 might play a role since in p53 mutated fibroblastic cell-lines derived from mice, spindle malformation and atypical mitotic figures were more common than in non p53 mutated cell-lines[14]. Accumulating evidence suggests that the interaction of HPV E6 and p53 inactivates wtp53 in HPV-infected epithelium and therefore provides the functional equivalent of p53 mutations[27].

Inhibition of p53 and pRB functions leads to deregulated cell growth and apoptosis and plays a central role in human carcinogenesis and tumor progression[21,29,30,31,32,48].

Wild type p53 has a short half life time and immunological detection of p53 in paraffin embedded sections is only possible in case of accumulation of truncated protein[26]. The mechanisms leading to accumulation of the protein in preinvasive lesions of cervical carcinoma are heterogeneous, and not only related to mutations. Nonmutational mechanisms for p53 accumulation include inactivation of enzymes responsible for the p53 degradation[11], stabilisation of p53 through complex formation with the product of MDM2 cellular gene[34,36], altered transcriptional regulation[36], and physiological accumulation during G1 and S phases of the cell cycle[19,41]. Unfortunately to date, immunohistochemical detection of p53 does not differentiate between upregulated wild type p53, mutated p53 or truncated p53. Nevertheless, several studies have documented a high correlation between p53 overexpression and mutations[8].

There are diverse data on p53 immunostaining of CIN. Bosari et al examined the immunolocalisation of p53 protein on formaline fixed parafin embedded cervical tissue with the monoclonal PAb 1801 and documented that suprabasal p53 immunoreactivity was observered in 25% of high-grade squamous intraepithelial lesions (CIN II and CIN

III) and in 72% of invasive squamous cell carcinomas. They also claimed p53 expression, though confined to the basal layer, in 74% of chronic cervicitis and in all cases of low-grade squamous intraeptihelial lesions[3].

Holm et al identified p53 expression in 7% of squamous cell carcinoma in situ (CIN III) and 62% of the invasive carcinomas using both monoclonal and polyclonal anti-p53 antibodies (PAb 1801 and CM1). They did not detect expression of p53 in normal, CIN I and CIN II lesions[22].

Jeffers et al stained 68 various grades of CIN lesions with sheep polyclonal antibody against p53. P53 expression was found in 13 out of the 22 CIN III cases, 3 out of the 14 CIN II cases and 5 out of the 13 CIN I cases. Suprisingly 7 out of 8 condylomata showed p53 expression. The stainings level was dichotomised in the presence of staining or no staining. The pattern of staining varied between the CIN grades; CIN II or less severe lesions in general showed staining of the basal or suprabasal layer whereas p53 stained througout the whole CIN III layer[25].

In 1995, Akasofu et al published a paper on p53 expression in 30 cases with metaplasia, 74 CIN lesions and 23 invasive carcinomas. CM1 antibody was used. P53 density per nucleus was scored on a three point scale and was summed up at the quantitivity of cells with p53 staining. They found no staining in CIN II or less lesions. CIN III and the invasive carcinomas scored in general low in p53 expression[1].

Ten Harmsel et al (1995), studied the intensity of p53 expression in 32 invasive carcinomas and 58 preinvasive lesions. They used the antibodies Bp63-12, 1801, DO7 and CM-1. The intensity of p53 expression was semi-quantitative scored into 4 classes. Preinvasive lesions showed weak to moderate p53 expression whereas all carcinomas demonstrated strong p53 expression[43]. However, p53 expression did not correlated with lymph node status and clinical outcome in 82 cases with cervical cancinoma, FIGO stage IB/IIA[24].

To summarize, CIN II or less severe lesions did not show p53 expression or just in the basal epithelial layer. This stands in contrast to our findings; we found in 6 out of the 12 CIN II lesions p53 expression. p53 expression in CIN III and invasive carcinomas was a common finding. None of these papers could demonstrate a relation between HPV type and p53 expression which is in concordance with our findings.

Most investigators, including ourselves, focussed their attention on the interaction

of E6 and p53, partly due the fact that p53 is readily accessible to immunohistochemical determination. This stands in contrast to the interaction of E7 and the retinoblastoma gene product. pRb is in a dormant state present in the cell. Activation of the pRb occurs by dephosphorylation. Detection of pRb by an immunohistochemical approach results in the detection of aswell the inactivated as the activate state of the protein and is therefore not informative.

In conclusion, P53 accumulation did not correlate with the presence of LTM, nor was p53 expression related to HPV type.

The role of p53 in the occurence of LTMs in CIN is questionable.

References

1 Akasofu M, Oda Y. Immunohistochemical detection of p53 in cervical intraepithelial lesions with or without infection of human papillomavirus types 16 and 18. Virch Arch 1995;425:593-602.

2 Baker SJ, Fearon ER, Nigro JN, Hamilton SR, Preisinger AC, Jessup JM, Van Tuinen P, Ledbetter DH, Barker DF, Nakamura Y., White R, Vogelstein B. Chromosomal 17 deletion and p53 gene mutations in colorectal carcinomas. Science 1989;244:217-21.

3 Bosari S, Roncalli M, Viale G, Bossi P, Coggi G. p53 Immunoreactivity in inflamatory and neoplastic diseases of the uterine cervix. J Pathol 1993;169;425-30.

4 Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 1995;87:796-802.

5 Burger MPM, Hollema H. The reliability of the histological diagnosis in colposcopically directed biopsies. A plea for LETZ. Int J Gynecol Cancer 1993;3:385-90.

6 Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predicitve value of human papilloma virus type for the histological diagnosis of women with two mild to moderately dyskaryotic cervical smears. BMJ 1995;310:94-95.

7 Burger MPM, van Leeuwen AM, Hollema H, Quint WGV., Pieters WJLM. Human papillomavirus type influences the extent of chromosomal lag during mitoses in cervical intraepithelial neoplasia grade III. Int J Gynecol Pathol 1997;16:10-4.

8 Busby-Earle RMC, Steel CM, Williams ARW, Cohen B, Bird CC. P53 mutations in cervical carcinogenesis-low frequency and lack of correlation with papillomavirus status. Br J Cancer 1994;69:732-7.

9 Carder P, Wyllie AH, Purdie CA, Morris RG, White S, Piris J, Bird CC. Stabilised p53 facilitates aneuploid clonal divergence in colorectal cancer. Oncogene 1993;8:1397-1401.

10 Chi CH, Rubio CA, Lagerlof B. The frequency and distribution of mitotic figures in dysplasisa and carcinoma in situ. Cancer 1977;39:1218-23.

11 Ciechanover A, DiGiusepe JA, Bercovich B, Orian A, Richter JD, Schwartz AL, Brodeur GM. Degradation of nuclear oncoproteins by the ubiquitin system in vitro. Proc Natl Acad Sci USA 1991;88:139-143.

12 Cooper K, Herrington CS, Evans MF, Gatter KC, O'D Mcgee J. P53 antigen in cervical condylomata, intraepithelial neoplasia and carcinoma: relationship to HPV infection and integration.

13 Cordon-Cardo C. Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 1995;147:545-559.

14 Cross SM, Sanchez CA, Morgan CA, Schimke MK, Ramel S, Idzerda RL, Raskind WH, Reid BJ. A p53-dependent mouse spindle checkpoint. Science 1995;267:1353-1356.

15 Crook T, Wrede D, Vousden KH. P53 point mutations in HPV negative human cervical carcinoma cell lines. Oncogene 1991;6:873-5

16 Duclic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JWE, Reed SI. P53 dependent inhibition of cycline dependent kinase activities in human fibroblasts during radiation induced G1 arrest. Cell 1994;76:1013-23.

17 Dustin P, Parmentier. Donnees experimentales sur la nature des mitoses abnormalis observees dans certains epitheliomas du col uterine. Gynecologie et Obstretrique 1953;52:258-65.

18 Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-67.

19 Helland A, Holm R, Kristensen G, Kaern J, Karlsen F, Trope C Nesland JN, Borresen Al. Genetic alterations of the TP53 gene, p53 protein expression and HPV infection in primary cervical carcinomas. J Pathol 1993;171:105-14.
20 Herrington CS. Human papilloma virus and cervical neoplasia. II. Interaction of HPV with other factors. J Clin Pathol 1995;48:1-6.
21 Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 mutation in human cancers. Science 1991;253:49-53.
22 Holm R, Skomedal H, Helland A, kristensen G, Borrensen A, Nesland JM. Immunohistochemical analysis of p53 protein overexpression in normal, premalignant, and malignant tissues of the cervix uteri. J Pathol 1993;169:21-6.
23 Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol 1993;13:775-84.
24 Hunt CR, Hale RJ, Buckley CH, Hunt J. P53 expression in carcinoma of the cervix. J Clin Pathol 1996;49:971-4.
25 Jeffers MN, Richmond J, Farquharson M, McNicol AM. P53 immunoreactivity in cervical intraepithelial neoplasia and non-neoplastic cervical squamous epithelium. J Clin Pathol 1994;47(12):1073-6.
26 Jensen RA, Page DL. p53: the promising story continues. Hum Pathol 1993;24:455-6.
27 Kessis TD, Slebos RJ, Han SM, Shah K, Bosch WF, Munoz N, Hedrikck L, Cho KR. P53 gene mutations and MDM2 amplification are uncommon in primary carcinomas of the uterine cervix. Am J Pathol 1993;143:1398-1405.
28 Kirland JA. Mitotic and chromosomal abnormalities in carcinoma in situ of the uterine cervix. Acta Cytol 1966;10:81-6.
29 Knudson AG. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci 1971;68:820-
30 Knudson AG. Retinoblastoma: in prototypic heriditary neoplasm. Semin Oncol 1978;5:57-9.
31 Knudson AG. Heriditary cancers: clues to mechanisms of carcinogenis. Br J Cancer 1989;59:661-6.
32 Levin AJ. The p53 supressor gene. New Engl J Med 1992;326:1350-2.
33 Melchers W, van de nBrule A, Walboomers J, De Bruin M, Burger M, Herbrink P, Meijer C, Lindeman J Quint W. Increased detection rate of human papillomavirus in cervical scrapes by the polymerase chain reaction as compared to modified FISH and Southern-blot analysis. J Med Virol 1989;2729-35.
34 Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The MDM-2 oncogene product forms a complex with p53 and inhibits p53-mediated transactivation. Cell 1992;69:1237-45
35 Mourits MJE, Pieters WJLM, Hollema H, Burger MPM. Three group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 1992;167:591-5.
36 Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 1992;358:80-3.
37 Parmentier R, Dustin P. Reproduction experimentale d'une anomalie particulier de la metaphase des cellules malignes ("metaphase a trois groupes"). Caryologia 1951;1:99-109.
38 Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW. The three-group metaphase as a morphologic indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 1992;14:227-232.
39 Poulsen HE, Taylor CW, Sobin LH. Histological typing of femal genital tract tumours. World Health Organization, Geneva 1975.

40 Scheffner M, Werness BA, Huibregtse Jm, Levine AJ, Howley AJ. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990;63:1129-36.
41 Shaulsky G, Ben-Ze'ev A, Rotter V. Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. Oncogene 1990;5:1707-11.
42 Snijders PJF, van den brule A, Schrijnemakers H, Snow G, Meijer CJLM, Walboomers JMM. The use of general primer in the PCR permits the detection of a broad spectrum of human papillomavirus infections. J Gen Virol 1990;72:2781-6.
43 Ten Harmsel B, Van Belkum A, Quint W, Pronk A, Kuijpers J, Ramaekers F, Tandon A, Smedts F. P53 and Human papillomavirus type 16 in cervical intraepithelial neoplasia and carcinoma. Int J Gynecol Pathol 1995;14:125-33.
44 Van den Brule AJC, Snijders PJF, Gordijn RLJ, Bleker OP, Meijer CJLM, Walboomers JMM. General primer mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. Int J Cancer 1990;45:644-9.
45 Van Leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and the mitotic index in cervical intraepithelial neoplasia. Virch Arch 1995;427:139-44
46 Van Leeuwen AM, Pieters WJLM, Hollema H, Burger MPM. Atypical mitotic figures and the mitotic index in microinvasive carcinomas and in non-neoplastic changes of the uterine cervix. J Obstet Gynecol 1996;16:269-76.
47 Vogelstein B, Kinzler KW. P53 function and dysfunction. Cell 1992;70:523-6.
48 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal tumor development. New Engl J Med 1988;319:525-32.
49 Winkler B, Crum CP, Fuji T, Ferenczy A, Boon M, Braun L, Lancaster WD, Richart RM. Koilocytotic lesions of the cervix. The relation of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 1984;53:1081-1087.

CHAPTER 7

HUMAN PAPILLOMAVIRUS IS RELATED TO DNA CONTENT IN CERVICAL INTRAEPITHELIAL NEOPLASIA

Abstract

Objective: To assess the relation between human papillomavirus type and increased DNA content in cervical dyskaryosis.

Study design: Cell suspensions obtained in a cross-sectional study on 345 patients with dyskaryotic cervical smears were analysed for human papillomavirus (HPV) types 6, 11, 16, 18, 31, and 33 by PCR and DNA content by DNA image cytometry. DNA cytometric analysis used single cell preparation. Based on nuclear size and density the number of cells with a DNA content exceeding 5C (N5C) were assessed and used as the main DNA cytometric parameter.

Results: HPV was found in 232 (67%) cases. The number of cells exceeding 5C (N5C) ranged from 0 to 2933 with a median of 17. HPV positive cases showed more N5C compared to HPV negative cases. Among the HPV positive cases, HPV-33 positive cases showed significantly more N5C. Cases harbouring an unkown HPV type showed comparable N5C to HPV negative cases.

Conclusion: The presence of HPV correlated to high numbers of cells exceeding 5C. Within the HPV positive cases HPV type 33 showed the highest number of cells exceeding 5C. HPV negative cases harboured in general low N5C.

Introduction

Cervical intraepithial neoplasia is a morphologically defined lesion associated with the development of cervical carcinoma. The severity is graded into CIN I, CIN II and CIN III, histologically paralleled by increasing nuclear atypia, increasing disturbance of the cellular differentiation and epithelial architecture, and increased mitotic activity[18]. High mitotic index, high numbers of lag type mitoses and high numbers of cells with a DNA content exceeding 5C are related to high grade CIN[22,31,32]. Normal diploid cells in mitoses have a maximum DNA content of 4C. Cells with a DNA content exceeding 5C will occure either during the DNA synthesis stage of cell division in peridiploid stemcells or, by multiple genetic events leading to endoreduplication in individual normal cells. Cells with a DNA content exceeding 5C are regarded as a marker for aneuploidy[6,23,29,30]. Aneuploid CIN lesions are likely to persist or progress to malignancy whereas diploid or polyploid lesions are expected to regress[1,9].

Human papillomaviruses are strongly associated with cervical neoplasia. Various types are found in CIN with type 16, 31 and 33 predominantly in CIN III[3,4,28] and 90% of the cervical carcinomas[2]. On epidemiological grounds, comparison of the strength of the association between specific HPV types in CIN and cervical carcinoma has indicated that type 31 and 33 have less malignant potential than type 16[17]. Arguments for a different malignant potential were also found in a previous study in which HPV type 16 positive CIN III cases showed significant higher number of lag type mitoses as compared to HPV-18 and HPV-31[5]. Moreover, HPV positive lesions were found to be aneuploid[37].

In this study, we evaluated the number of cells exceeding 5C in a series of patients with abnormal cervical smears. We investigated 1) the distribution of HPV types and the number of cells exceeding 5C in cervical scrapes of women with a dyskaryotic smear and 2) the relation of HPV type and the number of cells exceeding 5C.

Materials and methods

Patients

Patients were recruited from the colposcopy clinics of the Department of Gynecology of the University Hospital Groningen and the Martini Hospital Groningen. They were either referred by their general practitioner owing to an abnormal cervical cytology report, or the cervical cytological abnormality was discovered during gynecological examination. Patients were eligible for participation in the study if they had two smears indicating mild to moderate dyskaryosis or one smear indicating severe dyskaryosis. These indications correspond with the grounds for performing colposcopy as agreed by the cytopathologists and gynecologists in the Netherlands. In case of mild to moderate dyskaryosis the maximum interval between two abnormal smears was one year. Smears were not reviewed. Patients were excluded if 1) they had undergone previous colposcopical examination because of an abnormal cytology report; or 2) their cylindrical epithelium contained atypical cells or 3) they were pregnant. From 1 September 1988 to 1 September 1993, 345 consecutive patients fitted all criteria to enter the study. Out of the 345 cases, in 192 cases the cervix smear showed mild to moderate dyskaryosis and 153 cases showed severe dyskaryosis.

The age of the patients ranged from 19 to 69 years with a mean value of 35.5 (SD 8.6) years.

Sampling and processing of the cytological material.

The cervix was scraped with the blunt and pointed end of a wooden spatula and with an endocervical brush. The scraped cells were suspended in 5 ml phosphate buffered saline, pH 7.2, supplemented with merthiolate 1:10,000 v/v. This suspension was split into two samples. One sample, containing 2 ml, was analysed for the presence of HPV with the use of a general primer-mediated polymerase chain reaction[27] and type-specific primer-mediated polymerase chain reaction for the presence of HPV types 6, 11, 16, 18, 31, 33 seperately[19]. If the general primer PCR was positive but the type specific PCR was negative, the type remained unkown (designated "X"). The second sample, containing 3ml was resuspended in 10 ml polyonic ethanol, pH 7.4 and sent to Leiden, the Laboratory for Cytometry and Cytochemistry (Leiden University) for DNA analysis. The cell suspension was measured for cell density and, when needed, further diluted to about 20,000 (epithelial) cells/ml. After washing and fixing, the cell suspension was processed into a monolayer preparation by centrifugation techniques[30]. The monolayer preparations were stained with Feulgen Azur A. This allows the visual evaluation of abnormal cells in the samples. DNA measurements was performed with an image cytometry system[23]. This system enables automated screening of the slide, which encompasses selection of cells and measurements of its morphological and densitometrical parameters (DNA)[31]. The types of cells to be selected by the machine can be defined and programmed by the operator using a combination of size and density criteria. For the reported study we used criteria to select all types of epithelial cells, identified as random cell selection. After selection of 75 of these cells, more stringent criteria were applied to select abnormal cells exclusively. Further standard image processing include artefacts rejection and display of selected objects in a "gallery" on the TV screen[23]. This allows removing of the few remaining artefacts or overlapping cells not recognized as such by the system. DNA histograms therefore comprise single cell nuclei only. The median DNA content of 75 randomly selected cells was used as a diploid reference value. Subsequently the number of cells exceeding 5C in the whole slide of 1*1 cm was calculated. In some cases the machine had not screened the whole slide after selection of

250 cells. Then the number of cells was extrapolated to the estimated number in 400 microscopic fields, i.e. the entire area (1cm^2) of centrifuged cells.

Several DNA-content related parameters can be extracted from the DNA histogram of the population of abnormal cells. In a previous study we demonstrated that the most informative DNA related parameter was number of cells exceeding 5C among the population of abnormal cells[32].

Statistical analysis

The Kruskal-Wallis one-way analysis of variance was used to examine whether there was a significant difference in the distribution of the DNA parameter between the groups according to HPV type. The statistical and graphical procedures were performed with the SYSTAT software package[35]. P-values of less than or equal to 0.05 were considered to be significant.

Results

Distribution of HPV types and the cytological changes.

Table 1 shows the distribution of the various HPV types. Human papillomavirus was found in 232 (67%) of the cases. Only three cases harboured a mono-infection of HPV type 6 or 11. In 184 patients HPV types 16, 18, 31 or 33 were found. Twentyfive cases showed multiple HPV types and in 20 cases the general HPV primer was positive but the type could not be determined.

HPV was more often found in the severely dyskaryotic smears (80%(122/153)) compared to the mildly to moderately dyskaryotic smears (57% (110/192)) (chi-square test $p<0.001$).

Table I. The relation of HPV type and cervical dyskaryosis.

HPV type(s)	mild to moderate dyskaryosis	severe dyskaryosis	total
No HPV	82	31	113
6/11	2	1	3
6/11, 16	1	1	2
6/11, 16, 18	1	0	1
6/11, 16, 31	0	1	1
16	58	70	128
16, 18	4	2	6
16, 18, 31	0	1	1
16, 31	2	2	4
16, 33	2	0	2
18	9	12	21
18, 31	0	2	2
18, 31, 33	0	1	1
18, 33	2	1	3
31	7	10	17
31, 33	2	0	2
33	9	9	18
Unknown type	11	9	20
totals	192	153	345

Relation between the number of cells exceeding 5C and HPV types.

Among the 345 cases the number of cells with a DNA content exceeding 5C ranged from 0 to 2933 with a median of 17. The relation between N5C and HPV type is shown in figure 1. The median value (with limits of the interquartile range) of the N5C in the HPV positive and HPV negative group was 26.5 (4.5;94) and 4 (0;22), respectively. The presence of HPV resulted in a statistically significant higher number of cells exceeding 5C compared to HPV negative cases (Mann-Whitney U test $p<0.001$). We categorized the total population into 8 groups: those with an infection of HPV 6/11, 16, 18, 31 or 33

only, those with multiple HPV types, those with an unknown HPV type and those in whom no HPV was detected. Table II pressents the median value and limits of the interquartile range of N5C for each categories. We compared the distribution of N5C between those with an HPV type 16, 18, 31, 33 only, those with multiple HPV types and those with an unknown HPV type. The analysis revealed a significant difference regarding N5C between the six groups (Kruskall-Wallis one way of analysis p=0.02). The 18 cases with HPV 33 harboured a median value of 60 cells exceeding 5C.

Table II. N5C median and interquartile range per HPV type.

N5C	median	interquartile range	number of cases
HPV			
No HPV	4	0-22	113
16	37	5.5-120	128
18	21	9-71	21
31	20	5-40	17
33	60	24-118	18
mix	12	2-47	25
X	10.5	0-25	20
6/11 individual values: 1; 2; 186			3

Multiple HPV types were found in 25 (7%) cases. The cases harbouring multiple HPV types did not differ from the group with either a single HPV type or an unknown HPV type as concerns the N5C, age (Kruskall-Wallis one way of analysis p=0.08 and p=0.16 respectively) or severity of the cytological changes (p=0.47, chisquare test with Yates' correction)

Only three (0.8%) cases showed a mono-infection of type 6 or 11. Two patients had mild to moderate dyskaryosis and low numbers of cells exceeding 5C (1 and 2, respectively). The third patient was referred because of a severly dyskaryotic smear. High (186) number of cells exceeding 5C and HPV type 11 was found in the cervical scrape. The colposcopically directed biopsy showed CIN I.

An unknown HPV type was found in 20 cases. The distribution of the age, number of cells exceeding 5C (Kruskall-Wallis one way of analysis p=0.11 and p=0.42, respectively) or outcome of the cervical smear (p=0.19, chisquare test with Yates' correction) did not differ from the group patients **without** HPV infection. When we compared these 20 cases to the total group with known HPV types, no statistical differences was found with regard to age (Kruskall-Wallis one way analysis p=0.53) and severity of the cytological changes (chi-square test with Yates correction, p=0.63). A median N5C of 10.5 with an interquartile range of 0 to 25 was found which was statisticaly significant lower compared to the median N5C found in the total group of HPV positive cases (median 26.5, interquartile limits 6;104) (Kruskall-Wallis one way analysis, p=0.03)

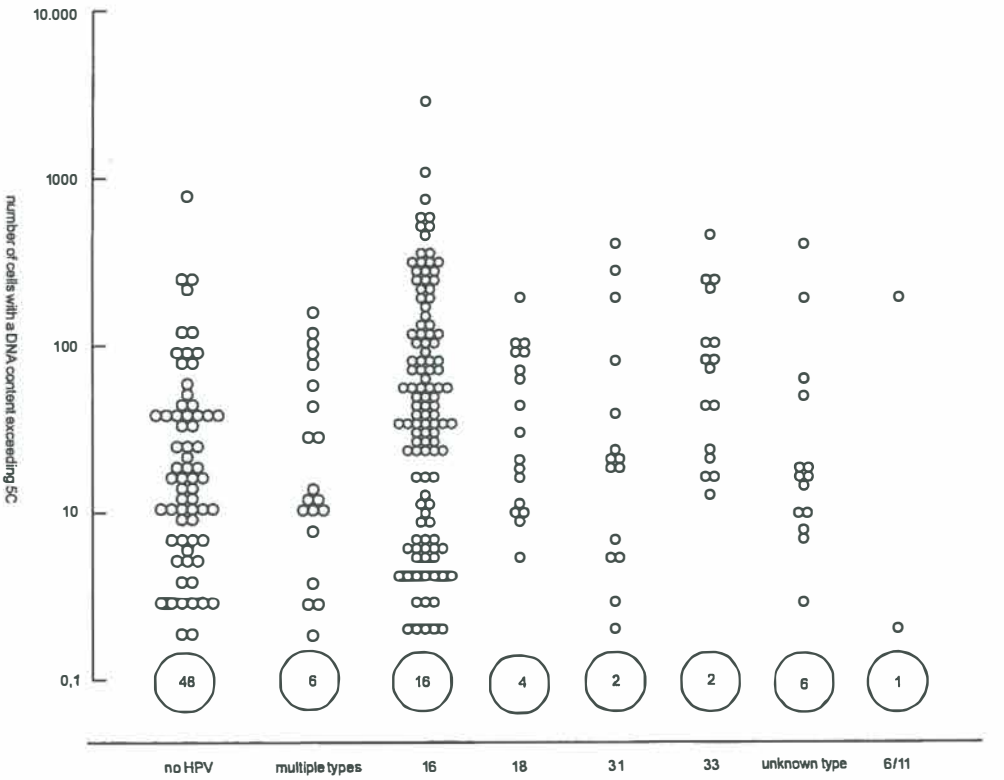


Figure I.: The relation between the number of cells exceeding 5C and human papillomavirus subtypes.

For graphical reasons 10log(N5C)=0 represents the number of cases demonstrating "0" and "1" cell exceeding 5C together.

We did not detect HPV in 113(33%) cases. The ages ranges from 20 to 69 years with a mean of 37.9 years which was statistically significantly older than the HPV positive cases (range 19-69 mean 34.5) (Mann-Whitney U-test p=0.01). The cytological smear indicated in 82(73%) mild to moderate dyskaryosis and in 30(27% severe dyskaryosis (chi-square test, p<0.001). The median of the number of cells exceeding 5C was 4 with a interquartile range of 0 to 32 (Table II).

Discussion

Human papillomavirus was found in 67% of the patients. All but three harboured "oncogenic" (type 16, 18, 31, 33 or a combination of these types) types and 20 showed an unknown HPV type. HPV positive cases showed more cells exceeding 5C than the HPV negative cases.

Within the HPV positive group significantly more cells exceeding 5C were found among the patients harbouring HPV type 33.

We performed this study on a large group of consecutively enrolled patients who were referred because of cytological changes indicating CIN. Only cytological data were taken into account because the patients were collected in three different hospitals and the histological work-up differed between the three hospitals.

DNA image cytometry and HPV typing were performed on aliquots of the same sample. The image cytometry method allows automated cell selection which is especially suited for reading cervical scrapes where the majority of the cells are normal and the abnormal cell populations is generally small. Visual cell selection is time consuming and subjective[6,34]. DNA distribution is usually expressed in diploid, polyploid or aneuploid histograms. The visual interpretation is hampered by interobserver variation. To limit this subjectivety, we used the number of cells exceeding 5C as an aneuploidy related parameter[30]. HPV typing was performed with the same set of primers[19]. Quality assurance programmes have indicated that the interpretation of HPV assays should be done with great care in order to avoid false-positive (and false-negative) assessments[24]. The quantity of the obtained material is also relevant since Guerrero demonstrated that the proportion of HPV positive women increased with increasing amount of cellular DNA in the samples[10,38].

Most of the HPV infections were found in the group of severely dyskaryotic smears which is in accordance with reports of other investigators[12,16,17,29]. The prevalence of genital HPV infection depends on the population examined and the HPV assay used[10,26]. We found nearly only oncogenic HPV types probably due to the stringent cytological entree criteria: all of the patients had either two mildly or moderately dyskaryotic smears or one severely dyskaryotic smear.

HPV positive cases harboured high numbers of cells exceeding 5C. No other reports were encoutered on the relation between cells exceeding 5C and HPV type. In some cases harbouring HPV 16 no cells exceeding 5C were found which was a intriguing finding since HPV and DNA cytometry was performed on the same sample. An explanation of this finding can be that the virus was an innocent bystander. High prevalence is reported among healthy young females. In a dutch study 25% of the women between 20 and 25 years old visiting their general practitioner because of oral contraceptives, cervical HPV infection was found[20].

The E6 proteins of HPV types 16, 31 and 33 show close similarity; these virus types belong to the same main branch of the phylogenetic tree which was constructed on the basis of the nucleotide and amino acid sequence alignment of the E6 gene[33]. The E6 protein of HPV-18 belongs to another main-branch. This HPV type was related to rapid-transit tumors[17], adenocarcinoma and small cell carcinomas. Moreover recent studies show that HPV type 18 is not a homogenous group as concerns oncogenic potential[11]. We found 21(6%) cases with HPV 18 which did not differ from the remaining HPV types with respect to the number of cells exceeding 5C. This stands in contrast to HPV type 33 lesions which showed the highest median number of cells exceeding 5C. In a previous study on 180 CIN III lesions, HPV 16 and 33 lesions showed statistically comparable number of lag type mitoses and level of mitotic index. In the latter report we concluded that this finding was coincidental because the group with HPV-33 cases was small[5]. In the epidemiological study done by Lorincz et al HPV-33 was classified as intermediate grade[17]. Despite the features of genetic instability (i.e. lag type mitoses and N5C) HPV-33 does not result in equal number of cervical carcinomas as does HPV-16.

We encountered three cases with HPV-6 or 11. Two of them showed, as expected, low numbers of cells exceeding 5C. One case harboured high number of cells exceeding

5C. Although HPV 6 and 11 are associated with benign infections, malignant transformation has been reported[25,36]. Whether the presence of HPV 6 or 11 is a coincidental finding in a non HPV related cervical carcinoma remains unclear.

We found that HPV negative patients were significantly older than HPV positive cases. This age dependency of HPV infection has been reported in groups of healthy women[8,20], women with dyskaryotic smears[4] and in cervical carcinoma patients[13].

Among the 113 HPV negative cases a substantial number harboured high numbers of cells exceeding 5C. This suggests that these lesions developed without the presence of HPV or that HPV infection was already resolved. Other genetically disturbing events for example p53 mutation may have occured. In vitro, HPV negative cell-lines have been found to harbour p53 mutations which is uncommon in HPV positive cellines[7]. In vivo substantial genetic alterations may occur without the presence of HPV[14,21]. Even an p53, HPV independent pathway is suggested[15].

In conclusion, HPV infection is related to high numbers of cells exceeding 5C. Also, HPV infection and high number of cells exceeding 5C are significantly more often found in severely dyskaryotic smears. Within the HPV positive group, HPV type 33 demonstrates high number of cells exceeding 5C. HPV negative cases show in general low numbers of cells exceeding 5C.

Aknowledgments

We thank professor dr. CJ Cornelisse for his critical comments.

We thank the gynecology and pathology departments of the Martini Hospital Groningen for their contributions made to the study.

Literature

1. Bibbo M, Dytch HE, Alenghat E, Bartels P, Wied GL. DNA ploidy profiles as prognostic indicators in CIN lesions. Am J Clin Pathol 1989;93:261-265.
2. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J et al. Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. J Natl Cancer Inst 1995;87:796-802.
3. Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predicitve value of human papillomavirus type for the histological diagnosis of women with two mild to moderately dyskaryotic cervical smears. BMJ 1995;310:94-95.
4. Burger MPM, Hollema H, Pieters WJLM, Schröder Quint WGV. Epidemiological evidence of cervical intraepithelial neoplasia without the presence of human papillomavirus. Br J Cancer 1996;73:831-836.
5. Burger MPM, Van leeuwen AM, Hollema H, WJLM Pieters. Human papilloma virus influences the extent of chromosomal lag during mitoses in cervical intraepithelial neoplasia grade III. Int J Gynecol Pathol 1997:16;10-14.
6. Cornelisse CJ, Van Driel-Kulker AM. DNA image cytometry on machine-selected breast cancer cells and comparison between flow cytometry and scanning cytophotometry. Cytometry 1985;6:471-477.
7. Crook T, Wrede D, Vousden KH. p53 point mutations in HPV negative human cervical carcinoma cell lines. Oncogene 1991;6:873-875.
8. De Villiers EM, Wagner D, Schneider A, Esch H, Munz F, Miklaw H, Zur Hausen H. Human papillomavirus DNA in women without and with cytological abnormalities: results of 5 years follow up study. Gynecol Oncol 1992;44:33-39.
9. Fu YS, Reagan JW, Richart RM. Definition of precursors. Gynecol Oncol 1981;12:220-231.
10. Guerrero E, Daniel RW, Bosch FX, Castellsague X, Munoz N, Gili M, Viladiu P, Navarro C, Martos C, Ascune N, Gonzalez LC, Tafur L, Izarzugaza I, Shah KV. Comparison of Virapap, Southern hybridization, and polymerase chain reaction methods for the detection of human papilloma virus (HPV) identification in an epidemiological investigation of cervical cancer. J Clin Microbiol 1992;30:2951-2959.
11. Hecht JL, Kadisch AS, Jiang G, Burk D. Genetic characterization of the human papillomavirus (HPV) 18 E2 gene in clinical specimens suggests the presence of a subtype with decreased oncogenic potential. Int J Cancer 1995;60:369-376.
12. Herrington CS. Human papilloma virus and cervical neoplasia. II. Interaction of HPV with other factors. J Clin Pathol 1995;48:1-6.
13. Higgins GD, Davy M, Roder D, Uzelin DM, Phillips GE, Burrell CJ. Increased age and mortality associated with cervical carcinomas negative for human papillomavirus RNA. Lancet 1991;338:910-913.
14. Kaebeling M, Burk RD, Atkin NB, Johnson AB, Klinger HP. Loss of heterozygosity on chromosome 17p and mutant p53 in HPV-negative cervical carcinomas. Lancet 1992;340:140-142.
15. Kessis TD, Slebos RJ, Han SM, Bosch XF, Munoz N, Hedrick L, Cho KR. P53 gene mutations and MDM2 amplification are uncommon in primary carcinomas of the uterine cervix. Am J Pathol 1993;143:1398-1405.
16. Kjaer SK, Van den Brule AJC, Bock JE, Poll PA, Engholm G, Sherman ME, Walboomers JMM, Meijer CJLM. Human papillomavirus. The most signigicant risk determination of cervical intraepithelial neoplasia. Int J Cancer 1996;65:601-606.
17. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papillomavirus infection of the cervix; relative risk association of 15 common anogenital types. Obstet Gynecol 1992;79:328-337

18. Poulsen HE, Taylor CW, Sobin LH. Histological typing of female genital tract tumours. World Health Organization, Geneva 1975.
19. Melchers DE, Van den Brule A, Walboomers J, De Bruin M, Burger M, Herbrink P, Meijer C, Lindeman J, Quint W. Increased detection rate of human papillomavirus in cervical scrapes by polymerase chain reaction as compared to modified FISH and Southen-blot analysis. J Med Virol 1989;27:329-335.
20. Melkert PWJ, Hopman E, Van den Brule AJC, Risse EKJ, Van Diest PJ, Bleker OP, Helmerhorst T, Schipper MEI, Meijer CJLM, Walboomers JMM. Prevalence of HPV in cytomorphologically normal cervical smears, as determined by polymerase chain reaction, is age dependent. Int J Cancer 1993;53:1-5.
21. Mullokandov MR, Kholodilov NG, Atkin RD, Johnson AB Klinger HP. Genomic alterations in cervical carcinoma: Losses of chromosome heterozygosity and human papillomavirus tumor status. Cancer Res 1996;56:197-205.
22. Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW. The three-group metaphase as a morphologic indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 1992;14:227-232.
23. Ploem JS, van Driel-Kulker AMJ, Ploem-Zaayer JJ. Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. Path Res Pract 1989;185:671-675.
24. Quint WGV, Heijtink RA, Schirm J, Gerlich WA, Niesters HGM. Reliability of methods for hepatitis B virus DNA detection. J Clin Microbiol 1995;33:225-228.
25. Schneider A, Kraus H, Schuhmann R, Gissman L. Papillomavirus infection of the lower genital tract: Detection of viral DNA in gynecological swabs. Int J Cancer 1985;35:443-448.
26. Schiffman MH, Bauer HM, Hoover RN, Glass AG, Cadell DM, Rush BB, Scott DR, Sherman ME, Kurman RJ, Wacholder S, Stanton CK, Manos MM. Epidemiological evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. J Nat Cancer Inst 1993;85:958-64.
27. Snijders PJF, van den brule A, Schrijnemakers H, Snow G, Meijer CJLM, Walboomers JMM. The use of general primer in the PCR permits the detection of a broad spectrum of human papillomavirus infections. J Gen Virol 1990;72:2781-6.
28. Van den Brule AJC, Snijders PJF, Gordijn RLJ, Bleker Op, Meijer CJLM, Walboomers JMM. General primer mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cerival scrapes and carcinomas. Int J Cancer 1990;45:644-649.
29. Van Driel-Kulker AMJ, Ploem-Zaayer JJ, van der Zwan-van der Zwan M, Tanke HJ. A preparation technique for exfoliated and aspirated cells allowing different staining procedures. Anal Quant Cytol 1980;2:243-246
30. Van Driel-Kulker AMJ, Ploem-Zaayer JJ. Image cytometry in automated cervix screening. Anal Cell Pathol 1989;1:63-77.
31. Van Leeuwen AM, Hollema H, WJLM Pieters, Burger MPM. Atypical mitotic figures and the mitotic index in cervical intraepithelial neoplasia. Virchows Arch (A) 1995;427:139-144.
32. Van Leeuwen AM, Ploem-Zaayer JJ, Pieters WJLM, Hollema H, Burger MPM. The suitability of DNA cytometry for the prediction of the histological diagnosis in women with abnormal cervical smears. Br J Obstet Gynecol 1996;103:359-365.
33. Van Ranst M, Kaplan JB, Burk RD. Phylogenetic classification of human papillomaviruses; correlation with clinical manifestation. J Gen Virol 1992;73:2653-2660.
34. Werstro RP, Liblit R, Koss LG. Flow cytometric DNA analysis of solid tumors: A review of the interpretations of DNA histograms. Hum Pathol 1991;33:1085-1098.
35. Wilkinson L. SYSTAT: The system for statistics. SYSTAT: Evanston, IL.

36. Wilczynski SP, Oft M, Cook N, Liao SY, Iftner T. Human papillomavirus type 6 in squamous cell carcinoma of the bladder and cervix. Hum Pathol 1993;24:96-102.
37. Winkler B, Crum CP, Fuji T, Ferenczy A, Boon M, Braun L, Lancaster WD, Richart RM. Koilocytotic lesions of the cervix. The relation of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 1984;53:1081-1087.
38. Woodman C. Epidemiology of HPV and cervical cancer. In Human papilloma virus and cervical cancer. Biology and immunology, Stern PL and Stanley MA.(eds.) pp.72-91. Oxford University Press: Oxford.

CHAPTER 8

THE SUITABILITY OF DNA CYTOMETRY FOR THE PREDICTION OF THE HISTOLOGICAL DIAGNOSIS IN WOMEN WITH ABNORMAL CERVICAL SMEARS

Summary

Objective: To analyse the suitability of DNA cytometry for predicting the histological diagnosis in women with cervical dyskaryosis.
Design: Survey with the use of diagnostic information to revise disease probability.
Setting: Colposcopy clinic of a university hospital.
Subjects: 110 patients with two mildly or moderately dyskaryotic cervical smears and 98 women with one severely dyskaryotic smear.
Interventions: DNA cytometric analysis using cytocentrifuge preparations of single cell suspensions from a cervical scrape. The main DNA cytometric parameter was N5C, i.e. the absolute number of cells with a DNA content of more than 5C on a given surface with a predefined cell density.
Main outcome measure: The probability of finding CIN II or worse. On arbitrary grounds, a positive test should point to a probability of 85% or higher.
Results: In the patients with cervical neoplasia, the value of N5C increased significantly with an increasing CIN grade ($p<0.001$). In the patients with one severely dyskaryotic smear and in those with two mildly or moderately dyskaryotic smears, the prior probability of finding CIN II or worse was 94% and 53%, respectively. Therefore, DNA cytometric analysis might be particularly useful in women with mild or moderate dyskaryosis; further analysis was restricted to this group. All of the women in whom the N5C value was higher than 52, were diagnosed as having CIN II or worse. Only 16 (14.5%) of the 110 women had an N5C value of 52 or higher. When the N5C value was 27, the probability of finding CIN II or worse was estimated to be 85%. Only 28 (25%) patients had an N5C value of 27 or higher.

Conclusions: DNA cytometry produced significant diagnostic information, as was shown by the relation between N5C and the histological diagnosis. However, the N5C value could not discriminate sufficiently between women with CIN II or worse and CIN I or better. Therefore, the management of individual patients with cytological abnormalities cannot be based on the results of DNA cytometric analysis.

Introduction

In recent years, electrosurgical loop excision of the transformation zone has tended to replace destructive techniques for the treatment of cervical intraepithelial neoplasia (CIN). Some gynaecologists prefer to treat a patient at her first visit. They argue that making a diagnosis by colposcopically-directed punch biopsies is of questionable accuracy and that loop electrosection of the transformation zone at the first visit is more economical[3,7,18]. This practice might lead to overtreatment, as cytological abnormalities (in particular mild and moderate dyskaryosis) may result from benign reactive changes[15,17]. There is an apparent need for non-morphological methods to predict the histological diagnosis. Some investigators have examined the predictive value of social criteria[4], smoking[19], or HPV type[13] in women with mild or moderate dyskaryosis. In the present study, we focused on the diagnostic information contained in the distribution of DNA content in morphologically abnormal cells.

The ploidy of a cell is assessed by counting the number of chromosomes or by measuring the DNA content. Cells are euploid if their nuclei contain two sets of 23 chromosomes (=2C) or a diploid amount of DNA (as assessed with a known reference). Polyploidy refers to a regular multiplicity of diploid values (4C, 8C, 16C, etc). Aneuploidy refers to an irregular number of chromosomes or DNA content, provided that the cell was not caught in the DNA synthesis (S) phase of the cell cycle.

We were particularly interested in aneuploidy, because it is a marker of malignancy[11]. In CIN, aneuploid lesions are likely to persist or progress to malignancy, whereas diploid or polyploid lesions can be expected to regress[6,14]. Some investigators have suggested DNA cytometry as a diagnostic tool in equivocal cases, in whom the goal is to differentiate between reactive inflammatory changes and true neoplasia[6,20].

The majority of cells in cervical scrapes are normal. In this situation, image cytometry is preferable because it allows cell selection. If image cytometry is performed, the ploidy of the histological lesion is inferred from the DNA histogram, i.e. the distribution of DNA content in multiple, selected (abnormal) cells. The visual classification of DNA histograms into diploid, polyploid or aneuploid is often subjective. An alternative ap-

proach is to use ploidy-related descriptors, such as the 5C-exceeding rate (5CER) and the 2C-deviation index (2CDI)[8,9,29]. The 5CER is the proportion of cells in the analysed population with a DNA content of more than 5C. The 2CDI is the mean square deviation between the DNA content in the selected cells and the diploid DNA content. Our laboratory also used the absolute number of cells with a DNA content of higher than 5C (N5C) in a cytological preparation with a given cell density[21]. This number can be corrected for polyploidy by subtracting the cells with a DNA content of 8C (N5C-8C).

In this study, we investigated whether the N5C, the N5C-8C, the 5CER and the 2CDI can predict the histological diagnosis in women with abnormal smears.

Materials and methods

Patients

The study group comprised 210 consecutive patients who were recruited from the colposcopy clinic of the Department of Gynaecology, University Hospital Groningen. They were either referred by their general practitioner owing to an abnormal cervical cytology report, or the cervical cytological abnormality was discovered during gynaecological examination. All of the patients had either two mildly or moderately dyskaryotic smears or one severely dyskaryotic smear. The cytological entry criteria corresponded with the grounds for performing colposcopy as agreed by cytopathologists and gynaecologists in the Netherlands. In the case of two smears showing mild or moderate dyskaryosis, the time interval between the taking of the smears was a maximum of one year. Patients were not included if they had previously undergone a colposcopic examination because of an abnormal cytology report, if their cervical smear indicated severe atypia of the endocervical cylindrical epithelium, or if they were pregnant.

The age of the patients ranged from 19 to 67 years with a mean value of 34.6 (SD 8.5) years.

Sampling and processing of the cytological material

The cervix was scraped with both the blunt and pointed end of a wooden spatula and with an endocervical brush. The scraped cells were suspended in 2 ml of phosphate buffered saline, pH 7.2, supplemented with merthiolate 1:10.000 v/v. This suspension was immediately mixed with 10 ml of polyonic ethanol, pH 7.4 and sent to the laboratory for DNA cytometry analysis. Each cell suspension was processed into a monolayer preparation by centrifugation techniques27. After washing and fixing, measures were included in the preparation procedure to control the cell density of the monolayers to about 20,000 (epithelial) cells/ml. The monolayer preparations were stained with Feulgen Azur A, which stains nuclear DNA quantitatively and is visually indistinguishable from Papanicolaou haematoxylin staining. This allowed the visual evaluation of abnormal cells in the samples.

DNA cytometry was performed with an image cytometry system (LEYTAS). This system performs automated cell selection based on nuclear density and size, and measures geometrical and densitometrical parameters (DNA)[28]. Standard routines of the equipment include artefact rejection tests and selected objects can be projected on to a TV screen[22]. The latter facilitates a final visual step to remove any remaining artefacts or overlapping cells from the data set. Histograms therefore comprise single cell nuclei only.

Firstly, the DNA content of 75 randomly selected cells was measured. As the majority of cells in a cervical smear will be normal epithelial cells, the mean integrated optical density (IOD) of the first peak of this cell population is the diploid (2C) reference population. Subsequently, the image cytometer was programmed with more strict density and size criteria to select any abnormal cells and to avoid selecting 2C cells. The criteria for selection were stepped up only moderately to include any cell nuclei with minor abnormalities. A maximum of 250 cells were measured and, if necessary, the number of cells was extrapolated to the estimated number in 400 microscopic fields, i.e. the entire area (1 cm^2) of centrifuged cells.

Four parameters were of interest on the DNA histogram of the population of abnormal cells: 1) the absolute number of cells with a DNA content exceeding 5C (N5C) in 400 microscopical fields; 2) the absolute number of cells with a DNA content exceeding 5C after subtracting the cells with a DNA content of 8C±0.8C (N5C-8C) in 400 microscopical fields; 3) the 5C-exceeding rate (5CER), which is defined as the proportion of

cells with a DNA content of more than 5C (in our notation, a proportion of e.g. 0.10 is equal to 10%); and 4) the 2C-deviation index (2CDI) which is defined as the mean square difference between the single cell DNA content and 2C.

Histomorphological examination

Four to 6 weeks after the cervix had been scraped to obtain cells for cytometrical analysis, we took colposcopically directed biopsies. If CIN was diagnosed in the biopsies, the whole transformation zone was excised about 6 to 10 weeks later by either loop electrosection or cold knife conization. Both treatment techniques removed the entire lesion for morphological examination. A diathermic loop was used if the entire neosquamocolumnar junction could be seen and it did not extend up into the endocervical canal for more than 5 mm, measured from the anatomical os externum. The details of the technique have been described previously[12]. We diagnosed and graded the cervical neoplasia according to the criteria of the World Health Organisation[23]. The histological sections were read independently by two pathologists. If there was a discrepancy between any of the readings, the sections were re-evaluated jointly and a consensus diagnosis was made. Neither of the pathologists were aware of the results obtained with DNA cytometry.

Statistical analysis

To test for a statistically significant difference between 2 or >2 groups regarding a quantitative variable, the Mann-Whitney U test or Kruskal-Wallis test was used, respectively. P values of ≤ 0.05 were considered to be significant.

In the clinical epidemiological analysis, the term 'prior probability' refers to the proportion of women with CIN II or worse, the term 'true-positive rate' to the proportion of women with CIN II or worse who have a positive test, and the term 'false-positive rate' denotes the proportion of women with CIN I or better who have a positive test. A ROC curve is a graph of the true-positive rate and the corresponding false-positive rate for each possible cut-off level of the diagnostic test result. ROC curves will be used to compare the discriminatory power of the ploidy-related descriptors. The most discriminatory descriptor is the most 'bowed', i.e. encloses the largest area under the curve[26]. If the

prior probability, the true-positive rate and the false-positive rate are known, the posterior probabilities can be calculated with the use of Bayes' theorem[1]. We use the term 'posterior probability' to denote the proportion of women with a positive test who have CIN II or worse. Posterior probabilities of ≥85% were considered to be clinically relevant. The alternative posterior probabilities, such as the probability for CIN I or better after a negative test or the probability for CIN II or worse despite a negative test, will be clearly indicated as such.

Results

In the 99 cases with severe dyskaryosis, the prior probability of finding CIN II or worse was 93/99 (94%). In the group of patients with two mildly or moderately dyskaryotic cervical smears, the prior probability of finding CIN II or worse was 59/111 (53%) (Table 1).

Table 1. Relation between the cytological and histological diagnoses

	cytological diagnosis		
histological diagnosis	mild to moderate dyskaryosis	severe dyskaryosis	total
no neoplasia	27	4	31
CIN I	25	2	27
CIN II	31	6	37
CIN III	27	83	110
carcinoma	1	4	5
total	111	99	210

In the total group of 210 patients, the number of abnormal cells ranged from 10 to 19600, with a median number of 433 and an interquartile range from 196 to 1027. Figure 1 shows the distribution on a binary logarithmic scale of the numbers of (machine-selected) abnormal cells in the population. We assessed the ploidy-related descriptors on the DNA histogram of the abnormal cells of each patient. The N5C ranged from 0 to 2933,

with a median value of 17 and an interquartile range from 3 to 68. The N5C-8C ranged from 0 to 2933, with a median value of 15 and an interquartile range from 2 to 54. The 5CER ranged from 0 to 0.57, with a median value of 0.05 and an interquartile range from 0 to 0.13. The 2CDI ranged from 0.10 to 14.09, with a median value of 2.99 and an interquartile range from 1.06 to 5.45.

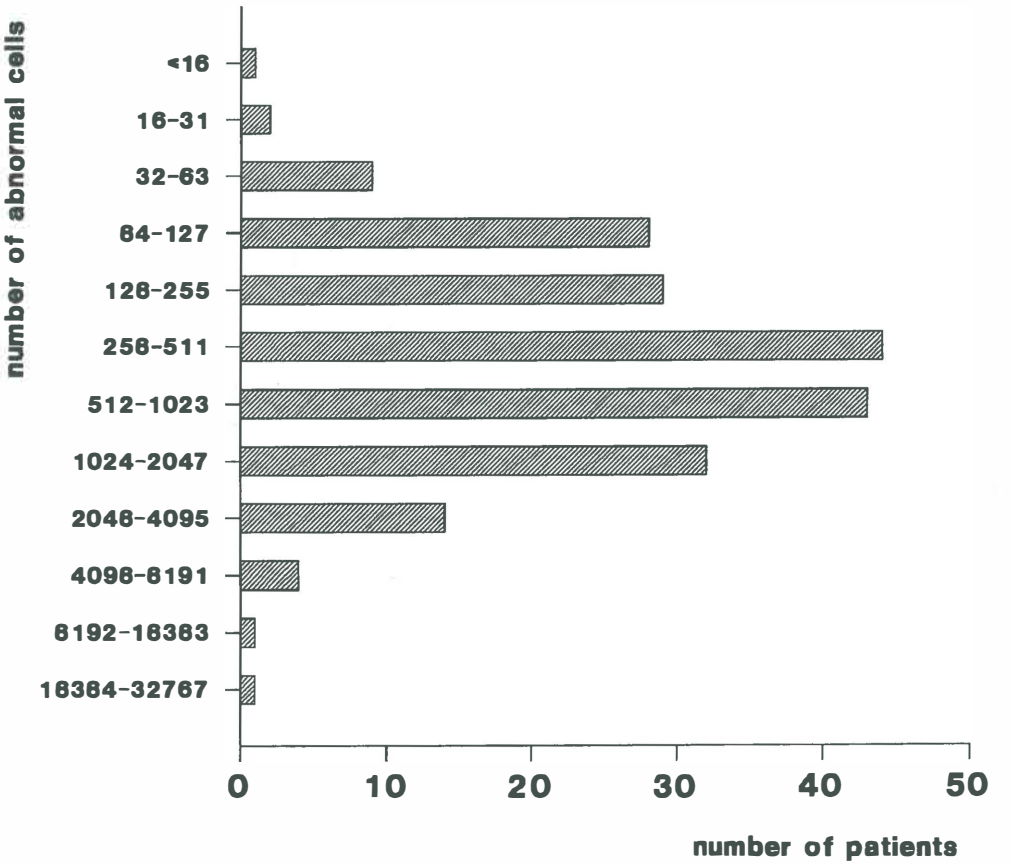


Figure 1: Distribution of the number of cytometrically-selected abnormal cells in the total population of 210 patients

We subsequently analysed the distribution of four ploidy-related descriptors per category of histological diagnosis. For the purpose of this analysis, we combined the 5

patients with (micro-)invasive carcinoma and those with CIN III into one group. In the group of 31 patients without cervical neoplasia, the value of N5C in 2 patients was incompatible with the rest of the data in this group. The first patient was referred because of two mildly dyskaryotic cervical smears. In the centrifuged preparation of cervical cells from this woman, we found 331 cells with a DNA content of more than 5C, which was in the upper part of the spectrum. Two colposcopically directed biopsies from this patient contained only mature metaplastic epithelium. Three consecutive follow-up cervical smears did not contain any abnormal cells. The second patient was referred because of severe dyskaryosis. In the centrifuged preparation of cervical cells from this woman, we found 104 cells with a DNA content of more than 5C, which was also in the upper part of the spectrum. No neoplasia were found in the colposcopically-directed biopsies, in the curettage specimen or in the cervical cone. These two patients obviously had outlying values of the DNA ploidy-related descriptors and were therefore excluded from further analysis.

Table 2 shows the median value and interquartile range values of N5C, N5C-8C, 5CER and 2CDI per histological category. The N5C, N5C-8C, 5CER and 2CDI values increased significantly with increased worsening of the histological changes (in all four instances $p<0.001$; Kruskal-Wallis tests). We note that the N5C, N5C-8C, 5CER and 2CDI values did not differ between the patients without neoplasia and the patients with CIN I ($p=0.07$, $p=0.12$, $p=0.16$ and $p=0.42$, respectively; Mann-Whitney U tests).

Table 2. Distribution of the ploidy-related descriptors per category of the histological diagnosis

histological diagnosis	N5C median (interquartile range)	N5C-8C median (interquartile range)	5CER median (interquartile range)	2CDI median (interquartile range)
no neoplasia	3 (0 - 21)	3 (0- 17)	0.01 (0.00-0.07)	1.57 (0.49-4.64)
CIN I	1 (0 - 80)	0 (0- 8)	0.00 (0.00-0.02)	1.49 (0.41-2.49)
CIN II	12 (5 - 33)	10 (4- 26)	0.04 (0.01-0.11)	2.85 (1.41-5.53)
CIN III	51 (6.5-132)	41 (6-111.5)	0.07 (0.03-0.17)	3.72 (1.91-6.34)

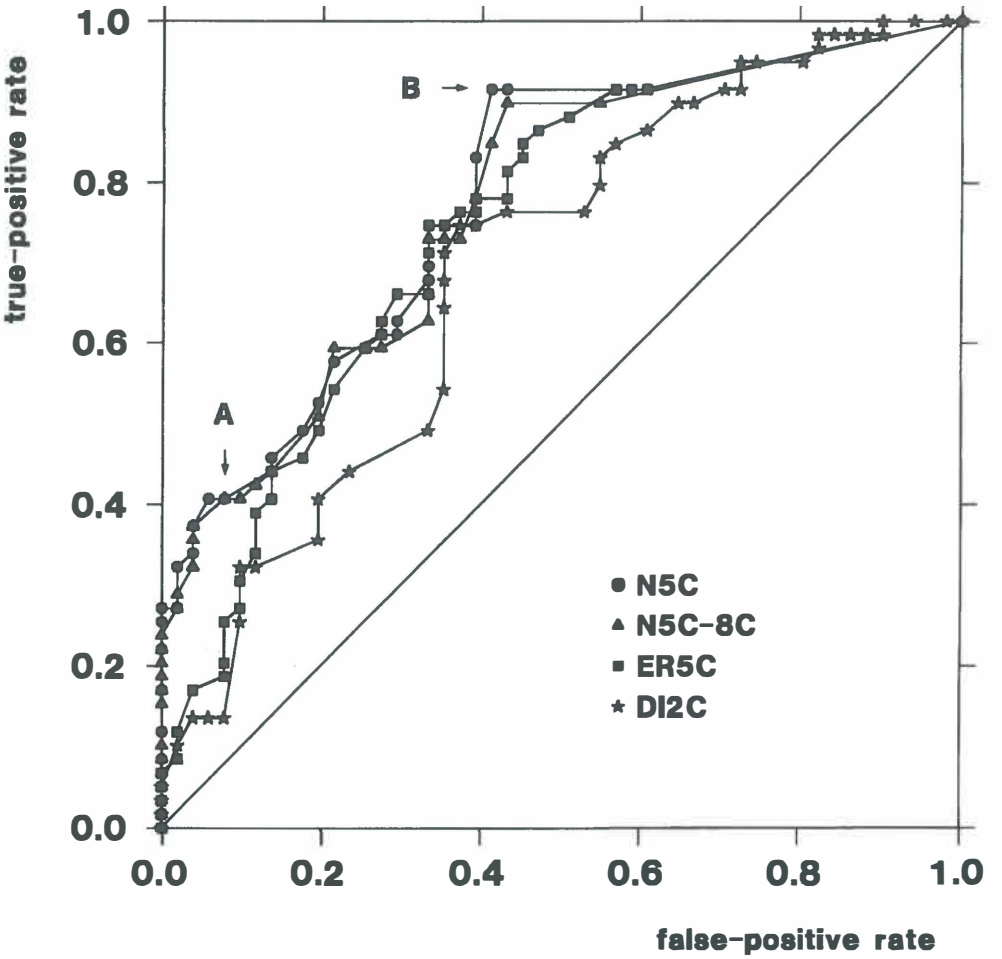


Figure 2: Relation between the rate of true-positive outcomes (CIN II or worse) and the rate of false-positive outcomes (CIN I or better) for any cut-off level of N5C, N5C-8C, 5CER and 2CDI (ROC-curves). The points 'A' and 'B' are discussed in the text.

DNA cytometric analysis might be clinically relevant for making a more accurate prediction of the histological diagnosis in patients with two smears showing mild or moderate dyskaryosis, because the prior probability of finding CIN II or worse in this group is only 53%. Further analysis was conducted on the 110 patients with this cytological diagnosis. The histological diagnoses were grouped into CIN I or better (n=51) and CIN II or worse (n=59). To analyse the discriminatory power of the ploidy-related descriptors, we drew ROC curves (Figure 2). A ROC curve shows the relation between the rate of true-positive outcomes (diagnosis CIN II or worse) and the rate of false-positive outcomes (diagnosis CIN I or better) for any cutoff level of ploidy-related descriptor. Figure 2 shows that the curve of N5C is the most 'bowed', i.e. courses nearest to the upper-left corner. Therefore, N5C has the most discriminatory power and we will focus on the N5C curve in the further analysis. The curve shows that the false-positive rate remains zero up to a true-positive rate of 27%. This point coincides with 52 cells with a DNA content of more than 5C. A total of 16 (14.5%) of the 110 women had an N5C value of 52 or higher. For any combination of true positive and associated false-positive outcomes, the posterior probability of finding CIN II or worse can be calculated given a prior probability of 53% (as was estimated in this study). At point 'A' in the N5C curve, which denotes a true-positive rate of about 40% (which means a test sensitivity of 40%) and an associated false-positive rate of about 8% (which means a test specificity of 92%), the posterior probability of finding CIN II or worse was 85%. This point coincides with the presence of 27 cells with a DNA content of more than 5C. A total of 28 (25%) out of the 110 patients had 27 cells or more with a DNA content >5C.

The other end of the spectrum of N5C values also contained diagnostic information. Figure 2 shows that at point 'B', which means at a true-positive rate (sensitivity) of about 90% and an associated false-positive rate of about 40% (and hence a specificity of 60%), the probability for finding CIN I or better following a negative test result is 84%. This figure also means that the probability that the patient does have CIN II or worse despite a negative test is 100-84=16%. Point 'B' coincides with the presence of 2 cells with a DNA content of more than 5C. A total of 35 (32%) out of the 110 patients had 2 cells or less with a DNA content >5C.

Discussion

Our study population comprised a comparatively large number of consecutively enrolled patients. We selected the patients in accordance with predefined entry criteria and diagnosed and treated them in accordance with the same management policy. Many studies on DNA cytometry have been performed on smears or biopsies from convenience samples of patients and healthy subjects. Consequently, it may be difficult to interpret what the results mean for everyday practice. We encountered two outlying values in the patients without cervical neoplasia. Spontaneous regression of the lesion might have occurred in these patients, but we may also have missed the lesion. According to a general guideline[2], we considered it to be reasonable to exclude these two cases from the further analysis.

The selection of cells for DNA cytometric analysis was fully automated. Visual cell selection has also produced useful results, but such a procedure is time-consuming, subjective and can be expected to be less reproducible. This applies particularly to the studies in which the investigators decided to exclude any cells with human papillomavirus-associated features from the analysis. There is substantial inter-observer variation in the reading of virus-induced changes[25]. Moreover, the large majority of CIN III lesions harbour HPV in the absence of morphological stigmata[24].

It is difficult to compare our results to those of other investigators. Although we used similar ploidy-related descriptors, our methods were different from those of other studies. We used a fully automated system to select cells from cytocentrifuge preparations, whereas other investigators selected cells visually from a restained cervical smear or from a particular area of a histological section. In our hands, the most discriminatory criterion was the absolute number of cells with a DNA content of >5C. A comparison of the absolute numbers between studies requires cytological cytocentrifuge preparations with a predefined cell density per square cm.

In our study, the cytometric findings in the women without neoplasia were similar to those in the women with CIN I. This finding is consistent with reports by pathologists that it is difficult to discriminate between benign reactive changes and CIN [15,16] stated that normal sections can be unequivocally distinguished from histological samples taken

from mild dysplasia by DNA cytometry. According to Böcking (1993)[10], benign and malignant lesions can be distinguished by the presence or absence of single aneuploid cells. Both Bibbo and Böcking performed their studies on convenience samples obtained from normal subjects. In contrast, we performed the analyses on women with cytological abnormalities, in whom we could not demonstrate cervical neoplasia. The different patient selection is probably responsible for the different conclusions.

The primary goal of our analysis was to evaluate whether the diagnostic information on a DNA histogram can be used for the management of patients with known cytological abnormalities. The prior probability of finding CIN II or worse in the patients with severe dyskaryosis was 94%, which is too high to make DNA cytometric analysis useful. In the patients with two mildly or moderately dyskaryotic smears, the prior probability was 53%. If we aim for a posterior probability of, for instance, 85% for having CIN II or worse, then we would need to perform a DNA cytometric test on four women in order to identify one patient. However, if we were to perform HPV typing, we could allot a posterior probability of 85% for having CIN II or worse to almost half of the patients[13]. At the other end of the spectrum of N5C values, we demonstrated that the probability for CIN II or worse in women who have ≤2 cells with a DNA content of >5C is estimated to be 16%. Although this probability is considerably lower than the prior probability of 53%, there remains an obvious indication for taking colposcopically-directed biopsies. Therefore, the practical importance of low N5C values in this patient population is limited.

We conclude that the results of DNA cytometry are not discriminative enough to justify its use as a guiding principle in the management of individual patients with cytological abnormalities. Nevertheless, the technique might prove to be useful in automated screening, because the combination of automated cell selection and DNA measurement might be an appropriate tool for identifying patients with a high risk for cervical neoplasia. Alternatively, the technique might be useful in women with borderline cytological changes to identify those with a low risk for this disease.

References

1. Altman DG (1991a). Practical statistics for medical research. Chapman & Hall, London, pp. 409-416.
2. Altman DG (1991b) Practical statistics for medical research. Chapman & Hall, London, pp. 126-130.
3. Anderson M. The role of LLETZ in the management of cervical intraepithelial neoplasia. In Large loop excision of the transformation zone (W. Prendiville, ed), (1993) Chapman and Hall, London, pp. 71-81.
4. Anderson DJ, Flannelly GM, Kitchener HC, Fisher PM, Mann EM, Campbell MK, et al. Mild and moderate dyskaryosis: can women be selected for colposcopy on the basis of social criteria? BMJ 1992;305:84-87.
5. Bibbo M, Bartels PH, Dytch HE, Wied GL Ploidy patterns in cervical dysplasia. Anal Quant Cytol Histol 1985;7:213-217.
6. Bibbo M, Dytch HE, Alenghat E, Bartels P, Wied GL. DNA ploidy profiles as prognostic indicators in CIN lesions. Am J Clin Pathol 1989;92:261-265.
7. Bigrigg MA, Codling BW, Pearson P, Read MD, Swingler GR. Colposcopic diagnosis and treatment of cervical dysplasia at a single clinic visit. Lancet 1990;336:229-231.
8. Böcking A, Adler CP, Common HH, Hilgarth M, Granzen B, Auffermann W. Algorithm for a DNA-cytophotometric diagnosis and grading of malignancy. Analyt Quant Cytol 1984;6:1-8.
9. Böcking A, Hilgarth M, Auffermann W, Hack-Werdier C, Fisher-Becker D, Von Kalkreuth G. DNA-cytometric diagnosis of prospective malignancy in borderline lesions of the uterine cervix. Acta Cytol 1986;30:607-615.
10. Böcking A. DNA Cytometry. In Surgical gynecologic oncology (E. Burghardt, ed), 1993 Thieme, New York, pp. 34-38.
11. Bohm N, Sandritter W. DNA in human tumors: a cytophotometric study. Curr Top Pathol 1975;60:151-219.
12. Burger MPM, Hollema H. The reliability of the histological diagnosis in colposcopically directed biopsies. A plea for LETZ. Int J Gynecol Cancer 1993;3:385-390.
13. Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predictive value of human papilloma virus type for histological diagnosis of women with two mildly or moderately dyskaryotic cervical smears. BMJ 1995;310:94-95.
14. Fu YS, Reagan JW, Richart RM. Definition of precursors. Gynecol Oncol 1981;12:S220-S231.
15. Hillier C. Loop diathermy excision. BMJ 1990;301:343.
16. Ismail SM, Colclough AB, Dinnen JS, Eakins D, Evans DMD, Gradwell E, et al. Observer variation in histopathological diagnosis and grading of cervical intraepithelial neoplasia. BMJ 1989;298:707-710.
17. Ismail SM. Loop diathermy excision. BMJ 1990;301:343.
18. Luesley DM, Cullimore J, Redman CWE, Lawton FG, Emens JM, Rollason TP, et al. Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears. BMJ 1990;300:1690-1693.
19. Luesley D, Blomfield P, Dunn J, Shafi M, Chenoy R, Buxton J. Cigarette smoking and histological outcome in women with mildly dyskaryotic cervical smears. Br J Obstet Gynaecol 1994;10:49-52.
20. Nasiell K, Näslund I, Auer G. Cytomorphologic and cytochemical analysis in the differential diagnosis of cervical epithelial lesions. Analyt Quant Cytol 1984;6:196-200.
21. Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW. The three-group metaphase as a morphologic indicator of high-ploidy cells in cervical intraepithelial neoplasia. Analyt Quant Cytol Histol 1992;3:227-232.

22. Ploem JS, Van Driel-Kulker AMJ, Ploem-Zaaijer JJ. Automated cell analysis for DNA studies of large cell populations using the LEYTAS image cytometry system. Path Res Pract 1989;185:671-675.
23. Poulsen HE, Taylor CW, Sobin LH. Histological typing of female genital tract tumours. World Health Organization, Geneva 1975.
24. Reid R, Crum CP, Herschman BR, Fu YS, Braun L, Shah KV, et al. Genital warts and cervical cancer. III. Subclinical papillomaviral infection and cervical neoplasia are linked by a spectrum of continuous morphologic and biologic change. Cancer 1984;60:1951-1959.
25. Robertson AJ, Anderson JM, Swanson Beck J, Burnett RA, Howatson SR, Lee FD, et al. Observer variability in histopathological reporting of cervical biopsy specimens. J Clin Pathol 1989;42:231-238.
26. Sackett DL, Haynes RB, Tugwell P. Clinical Epidemiology: a basic science for clinical medicine. Little, Brown and Company, Boston/Toronto, 1985 pp. 59-138.
27. Van Driel-Kulker AMJ, Ploem-Zaaijer JJ, Van der Zwan-Van der Zwan M, Tanke HJ. A preparation technique for exfoliated and aspirated cells allowing different staining procedures. Anal Quant Cytol 1980;2:243-246.
28. Van Driel-Kulker AMJ, Ploem-Zaayer JJ. Image cytometry in automated cervix screening. Anal Cell Pathol 1989;1:63-77.
29. Wagner TMU, Adler A, Sevelda P, Assmann I, Knepflé CF, Czerwenka K, et al. Prognostic significance of cell DNA content in early-stage ovarian cancer (FIGO stages I and IIa) by means of automatic image cytometry. Int J Cancer 1994;56: 167-172.

CHAPTER 9

GENERAL DISCUSSION AND SUMMARY

General discussion and summary

In this thesis, we have described studies regarding cervical (intraepithelial) neoplasia. More specifically, we were interested in reproducible quantitative features to distinguish the various grades of intraepithelial neoplasia (CIN) and whether these features contributed to our knowledge about the etiology of CIN. We have focused on proliferation rate, the occurrence of atypical mitotic figures, infection with human papillomavirus and cellular DNA content. These items will be discussed first and the relevant chapters will be summarized.

Atypical mitotic figures and the mitotic index.

The presence of abnormal mitotic figures and an increased proliferation rate are histomorphological features which are commonly found in cervical carcinomas. Atypical mitotic figures and an increased proliferation rate support the diagnosis of CIN. We examined whether these features could be used as a reliable quantitative diagnostic criterium for those CIN lesions which may be at risk for future invasion. In chapter 2, we focused on the mitotic index (MI) and the occurrence of normal and atypical mitotic figures in cervical intraepithelial neoplasia in a series of 127 women with dyskaryotic cervical smears. In the area with the highest number of mitoses, the mitotic index was assessed. The median value of the mitotic index increased from 3 in CIN I lesions, through 4 in CIN III to 9 in CIN III lesions. In chapter 3, we studied nonneoplastic changes and micro invasive carcinoma. In the CIN III lesions adjacent to the micro invasive part and in the micro-invasive carcinoma the median value of the MI (median value MI: 7.5; 7 respectively) was comparable to the median value of 9 in the CIN III lesions not adjacent to invasive carcinoma (chapter 2). This was also reported by Chi et al who studied the percentage of mitoses in 83 CIN lesions and 25 micro invasive carcinomas. They found increasing percentages of mitoses with increasing CIN grade. CIS, CIS adjacent to MIC and MIC demonstrated comparable (0.6(±0.3), 0.7(±0.3) and 0.6(±0.1), respectively) percentage of mitoses[8]. These findings suggest that the mechanisms resulting in dysregulation of cell proliferation expressed as an increased MI do not alter upon the point of invasion. Despite the unaltered cellproliferation, the tumor volume increases. This might result from a decrease of programmed cell death, apoptosis[7]. Apoptosis is influenced by

cellular chemical alterations such as hypoxia or withdrawal of trophic hormones from target tissue like prostate atrophy after castration. Paradoxically, c-myc as well can influence apoptosis and stimulate proliferation. Moreover, the tumorsupressorgene p53 can stimulate apoptosis. In cervical carcinoma, p53 function can be impaired by interfering with the human papillomavirus product E6[20] whereas c-myc expression can be disturbed by the integration of HPV close to the site of this gene[10].

Next to the assessment of the MI, we analysed the occurrence of normal and atypical mitotic figures. This was examined in the same area which was used for the assessment of the MI. We distinguished lag type mitoses, multipolar mitoses, other atypical mitotic figures and normal mitoses. Multipolar mitoses are metaphases with an abnormal configuration of the equatorial plate; the chromosomes are located along several radial axes. The tripolar mitoses and the quadripolar mitoses are the most common. Lag type mitoses are mitoses with non-attached condensed chromatin in the area of the mitotic figure. The three group metaphase (3GM) and the two group metaphase (2GM) are the two most easily recognized.

In our series of CIN lesions (chapter 2), we found increasing numbers of lag type mitoses and decreasing numbers of normal mitoses with increasing CIN grade. We could not demonstrate a relation between the number of other atypical mitotic figures and CIN grade. The nonneoplastic changes (chapter 3) exhibit almost no lag type mitoses or multipolar mitoses. Normal and other atypical mitotic figures occurred frequently. In the CIN III lesions adjacent to carcinoma and micro-invasive carcinoma (chapter 3), normal and other atypical mitotic figures occurred frequently whereas multipolar mitoses were scarce. Lag type mitoses were present in all CIN III lesions adjacent to the micro-invasive part and in micro invasive carcinomas. The highest median value of the number of LTMs occurred in the CIN III lesions adjacent to the micro invasive part.

Previous studies have shown that lag type mitoses especially the 3GM, are related to aneuploidy[22] and large numbers of high ploid cells[18]. This suggests that LTM is a morphological expression of genetic instability. Micro-invasive carcinomas exhibit fewer lag type mitoses than adjacent CIN lesions and usually are morphologically better differentiated than CIN III lesions indicating a genetically more stable population. Not all CIN III lesions progress to invasive carcinoma. Within the total population of CIN III lesions, subpopulations develop which are capable of invasion. We suggest that

the occurrence of subpopulations with an infiltrating potential, is paralleled by a decrease of lag type mitoses.

Some CIN III lesions demonstrated low MI (less than 3) and low numbers of, or no lag type mitoses. These lesions showed epithelial architecture and cellular polymorphism which was severe enough to classify the lesion as CIN III, despite the scarcity or absence of mitoses. We suggest that these lesions have a different biological potential.

In some CIN II lesions the number of lag type mitoses was comparable to the number of lag type mitoses in CIN III lesions adjacent to invasive carcinoma. This suggests an invasive potential. Whether CIN II lesions can become invasive carcinomas without going through the stage of CIN III is a matter of debate. Morphologically, this might be reflected by the presence of lag type mitoses.

The suitability of using the occurrence of lag type mitoses in conjunction with an elevated MI as a diagnostic test for the preinvasive cervical lesions was described in chapter 4. The traditional CIN classification, subdivided into three grades, was compared to the quantitative model. This model was based on an MI of 3 or more in conjunction with the presence of 2GM or 3GM. In a series of 199 patients the biopsy diagnosis was compared to the most severe lesions found in the whole transformation zone. An under diagnosis in the biopsy specimen was more often made by using the quantitative model. A second disadvantage of the quantitative model is the fact that relatively large biopsies are needed to enable the classification of 228 mitoses. Therefore, we concluded that counting and analysing mitotic figures alone is not suitable for daily diagnostic routine. It may be helpful for discriminating CIN from benign reactive changes. Although treating CIN I or II lesions is a matter of discussion, we suggest that the lower CIN grades showing an MI of 3 or more in conjunction with two or three group metaphases should be considered for treatment.

Human papillomavirus

Human papillomavirus is present in more than 90% of cervical carcinoma[4]. HPV is found in CIN lesions with a predominance of types 16, 31 and 33 in CIN III[5]. In chapter 5 and 6 we describe studies on lag type mitoses and the mitotic index in relation to human papillomavirus type (chapter 5) and to p53 expression (chapter 6). We found that

CIN III lesions with HPV-16 or HPV-33 showed more LTMs compared to HPV-18 and HPV-31. CIN III lesions with HPV-16 demonstrate higher values of the MI compared to the other HPV types (chapter 5). Since only cases with CIN III lesions were studied, the relevance of lag type mitoses and HPV type in distinguishing the various CIN grades was beyond the scope of this study.

With respect to the etiological relevance, human papillomavirus is strongly correlated to cervical carcinoma and its precursor lesions. HPV type 16 is the predominant type found in cervical neoplasia. HPV has a proliferation inducing capacity. This is found for all HPV types since induced cell proliferation by HPV-6 or HPV-11 infection results in condylomata acuminata in the cervix. Besides the proliferation induction the E6 protein of HPV 16, 18, 31 and 33 has an oncogenic function since it binds to p53 and thereby inhibiting its function. P53 has several checkpoints in the cell cycle. The G1 checkpoint is the most well-known and allows cells time to repair DNA damage. Recently, it has been shown that p53 mutated fibroblastic celllines from mice showed mitotic arrest, multiple centrioles and more atypical mitotic figures. These findings resulted in the suggestion that p53 may act as a mitotic checkpoint[11]. The genesis of lag type mitoses is not fully understood but LTMs may arise because of spindle malformation[17] or abnormal distribution of highploid DNA[16,18]. So, p53 may play a role in the development of lag type mitoses. The latter observation was the rationale for the study described in chapter 6. In a series of 74 patients, we studied the occurrence of LTMs and the expression of p53. No relation could be demonstrated between LTMs and p53 expression, nor was p53 expression related to CIN grade or HPV-type. There are several data on p53 immunostaining. Bosari et al (1993)[3] examined the immunolocalisation of p53 protein on formalin fixed paraffin embedded cervical tissue with the monoclonal PAb 1801 and documented that suprabasal p53 immunoreactivity was observed 25% of high-grade squamous intraepithelial lesions (CIN II and CIN III) and 72% of invasive squamous cell carcinomas. They also claimed positive p53 expression though confined to the basal layer, in 74% of chronic cervicitis and in all cases of low-grade squamous intraepithelial lesions. Holm et al (1993), identified p53 expression in 7% of squamous cell carcinoma in situ (CIN III) and 62% of the invasive carcinomas using both monoclonal and polyclonal anti-p53 antibodies (PAb 1801 and CM1). They did not detect expression of p53 in normal, CIN I and CIN II lesions[13].

Jeffers et al (1994), stained 68 various grades of CIN lesions with sheep polyclonal

antibody against p53. P53 expression was found in 13 out of the 22 CIN III cases, 3 out of the 14 CIN II cases and 5 out of the 13 CIN I cases. Surprisingly, 7 out of 8 condylomata showed p53 expression. The staining level was dichotomized in the presence of staining or no staining. The pattern of staining varied between the CIN grades; CIN II or less severe lesions in general showed staining of the basal or suprabasal layer whereas p53 stained throughout the whole CIN III layer[14].

In 1995, Akasofu et al published a paper on p53 expression in 30 cases with metaplasia, 74 CIN lesions and 23 invasive carcinomas. CM1 antibody was used. P53 density per nucleus was scored on a three point scale and was summed up at the quantity of cells with p53 staining. No staining was found in CIN II or less lesions. CIN III and the invasive carcinomas scored in general low in p53 expression[1].

To summarize, CIN II or less severe lesions did not show p53 expression or just in the basal epithelial layer. P53 expression in CIN III and invasive carcinomas was a common finding. None of these papers demonstrates a relation between HPV type and p53 expression which is in concordance with our findings. Mutated p53 in cervical carcinoma is found in a low prevalence[6] and therefore, we assume that most of the immunohistochemically detected p53 in CIN is wild type p53. We question the importance of p53 expression detected by immunohistochemistry in the carcinogenesis of cervical neoplasia.

Cellular DNA content

Abnormal amount of cellular DNA content (aneuploidy) is one of the features of most malignancies. In premalignant cervical lesions, aneuploid lesions progressed more often to higher grades than eu- or polyploid lesions[12]. Chapter 7 and 8 describe studies on aneuploid cervical smears. As a descriptor for aneuploidy, we used the number of cells with a DNA content exceeding 5C. In chapter 7, we studied the number of cells exceeding 5C in relation to human papillomavirus in dyskaryotic cervical smears in order to get insight in the influence of HPV infection in the development of abnormal DNA content. We found significantly more cells exceeding 5C in sever dyskaryotic smears compared to the mild to moderate dyskaryotic smears and significantly more cells exceeding 5C in the HPV positive cases compared to the HPV negative group. Within the HPV positive group, HPV-33 harbored most cells exceeding 5C. HPV-33

together with HPV-18 and HPV-31 is usually regarded as an intermediate risk type[15]. But judged from its ability to induce high number of cells with a DNA content exceeding 5C and high numbers of lag type mitoses (chapter 5), HPV-33 has a biological profile comparable to HPV-16. HPV-33 was found in the same phylogenetic main branch as HPV-16 but together with HPV-31 and HPV-18 on a different subbranch of the phylogenetic tree. This tree is based on the alignment of the nucleotide sequence of the E6 gene[21]. This different biological profile of HPV-33 did not result in high numbers of HPV-33 positive carcinomas[15]. An explanation can be that HPV-33 infection induces a more aggressive immunological response and will thereby be resolved before invasive carcinoma can occur.

Chapter 8 describes a study with a different approach compared to the former chapters. We used DNA related descriptors such as the number of cells exceeding 5C, number of nonpolyploid cells exceeding 5C, 5C exceeding rate and 2C deviation index as screening parameters in a large group of consecutive collected women with abnormal cervical smears in order to predict the histologic diagnosis. All DNA related descriptors were (statistically significant) positively correlated to CIN grade. In this study the prior probability of CIN II or worse in the group of patients with severly or mildly to moderately dyskaryotic smears was 94% and 53%, respectively. Therefore, we focused on the cases with mild to moderate dyskaryosis and studied whether DNA cytometry could contribute to the prediction of lesions with CIN II or more. To express the discriminatory power of these variables, we used a ROC (receiver operating characteristic) curve. A ROC curve is a graph of the true positive rate and the corresponding false positive rate for each possible cutoff level of the diagnostic test result. The most discriminatory descriptor produces a curve at the upper left corner of the graph and thus has the largest area under the curve[19]. In our series, the number of cells exceeding 5C showed the most bowed graph compared to the number of nonpolyploid cells exceeding 5C, the 5C exceeding rate and the 2C deviation index. We found that cervical cell suspensions containing 52 cells exceeding 5C or more all demonstrated CIN II or more. However, this was only present in 16 out of the 110 cases (14%). Moreover in the cases with 2 or less cells exceeding 5C the probability on CIN II or higher was still 16%. Therefore, in our hands and in the study setting as described, the practical importance of DNA cytometry in daily routine proved to be limited.

The aim of this thesis was to study reproducible quantitative features to distinguish the various grades of CIN and to examine whether these features contributed to our knowledge about the etiology of CIN. We concluded that analysing atypical mitotic figures and mitotic index is a helpful feature to diagnose CIN but unfortunately the CIN classification can not be replaced by the quantification of mitotic figures alone. We suggest, however that lower CIN grades showing an MI of 3 together with the presence of lag type mitoses should be considered for treatment.

The development of cervical carcinoma and its precursor lesions is correlated to abnormal cellular DNA content and infection of oncogenic HPV types. In this process p53 may play a role.

Moreover, the practical importance of DNA cytometry in daily routine is limited.

Prospects

In this thesis we described studies on cervical intraepithelial neoplasia. The morphological classification of CIN is hampered by a high intra and interindividual variation. Moreover, this morphological approach provides not enough information to differentiate between lesions that will regress or progress to invasive cancer. We tried to improve the morphological classification by introducing the presence of lag type mitoses as an additional morphological criterium. As our studies show, it did not result in a better classification.

It has been shown that CIN lesions are heterogenous and multifocal. This provides problems if a diagnosis is based on biopsy alone. It may be necessary to study the entire lesion before a definite diagnosis can be made. This provides a fundamental problem to any method applied using only a part of the CIN lesion. It is not very likely that in the future additional morphologic studies will solve the problems with the CIN classification.

The boom in DNA technology has resulted in a better understanding of the genesis of the phenotype of malignant and premalignant lesions. In chapter 7 and 8 we measured the amount of DNA in individual cells which is a relatively crude method. Karyotyping of metaphases spreads allows the detection of abnormal number of chromosomes and translocations. Culturing dysplastic epithelial cells may be difficult

and in the literature no reports on karyotyping on CIN are present. It may be more easy to study individual chromosomes in CIN by in situ hybridization using chromosome specific probes. A more sensitively technique is comparative genomic hybridization (CGH) whereby metaphase chromosomes are used for chromosomal gains and losses. More specifically, by this technique normal metaphases chromosomes are competitively hybridized with two differentially labeled genomic DNAs: test and reference DNA whereby the differences in copy number are detected between the two DNAs. With this technique, we could examin the DNA alteration in the flanking DNA regions of known oncogenes or tumor supressor genes.

Genetic aberrations of nonmitotic DNA can be found by the technique of loss of DNA heterozygosity (LOH). By this technique, allelotypes of normal and tumor DNA can be compared whereby the loss of specific alleles may point to an important DNA region which may harbour tumor supressor genes. In cervical carcinoma LOH is frequently found on the short arm of chromosome 3 and 6 indicating the presence of tumor supressor genes which may be involved in the pathogenesis of cervical carcinoma. These studies could be continued to examin whether specific chromosomal regions are involved in the progression from normal cervical epithelium through intraepithelial neoplasia to invasive carcinoma.

Human papillomaviruses (HPV) play a keyrole in the development of cervical cancer. The proteins encoded by the introns E6 and E7 interfere with the p53 protein and the protein encoded by the retinoblastoma gene. Highly sensitive techniques such as PCR detect HPV, especially the E6 intron, in more than 90% of the squamous cell carcinomas. Some authors advocate the introduction of HPV detection in screeningsprotocols.

An interesting possibility is the vaccination of healthy young women against HPV. This might prevent the development of CIN and thereby cervical cancer.

Another possibility is in vitro priming of cytotoxic T-cells followed by intralesional injection

It is generally known that psychosocial stressors interfere with health; unhappy people are more susceptive of disease. By prospective epidemiological studies, we could study whether high psychosocial stressors are related to the development or progression

of CIN and whether high psychosocial stressors are related to HPV load by for example HPV hybrid capture method.

Many CIN lesions show a high mitotic index and a high apoptotic rate. Apoptosis can be increased by hypoxic states, viral infection or aberrant DNA. P53 has an important keyrole in the induction of apoptosis but its function is impaired by binding to HPV-E6 protein. The balance between apoptosis and proliferation seems to be different in invasive carinomas compared to adjacent CIN lesions. This a partly due because cervical epithelium obtains its nutrients and oxygen by diffusion from subepithelial vessels whereas invasive carcinomas process can grow out because of the induction of angiogenesis. Little is known about the regulation of apoptosis in CIN and carcinomas. Quantification of proteins involved in apoptosis regulation may lead to a better understanding of this process.

The lifespan of individual cells is limited by the length of its telomeres. Telomeres shorten during every cellcycle and at a critical length the cell will either die or go into senescence. Telomerase elongates the telomeres whereby cells enhance their lifespan and may become immortal. Most malignant cells demonstrate upregulation of telomerase. HPV and Ebstein-Barr virus interfere with telomere length. By comparative studies on telomerase activity in cervical intraepithelial neoplasia and early invasive carcinoma may indicate which CIN lesions progress to invasive carcinoma.

The etiology of cervical neoplasia starts to be unraveled, but we are still not able to predict the clinical outcome in a individual patient.

References

1. Akasofu M, Oda Y. Immunohistochemical detection of p53 in cervical intraepithelial lesions with or without infection of human papillomavirus types 16 and 18. Virch Arch 1995;425:593-602.
2. Baker SJ, Fearon ER, Nigro JN, Hamilton SR, Preisinger AC, Jessup JM, Van Tuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B. Chromosomal 17 deletion and p53 gene mutations in colorectal carcinomas. Science 1989;244:217-21.
3. Bosari S, Roncalli M, Viale G, Bossi P, Coggi G. p53 Immunoreactivity in inflamatory and neoplastic diseases of the uterine cervix. J Pathol 1993;169;425-30.
4. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 1995;87:765-802.
5. Burger MPM, Hollema H, Pieters WJLM, Quint WGV. Predictive value of human papillomavirus type for histological diagnosis of women with cervical cytological abnormalities. BMJ 1995:310:94-5.
6. Busby-Earle RMC, Steel CM, Williams ARW, Cohen B, Bird CC. p53 mutations in cervical carcinogenesis-low frequency and lack of correlation with human papillomavirus status. Br J Cancer 1994;69(4):732-7.
7. Carson D, Ribeiro JM. Apoptosis and disease. The Lancet 1993;341:1251-4.
8. Chi CH, Rubio CA, Lagerlof B. The frequency and distribution of mitotic figures in dysplasisa and carcinoma in situ. Cancer 1977;39:1218-23.
9. Cordon-Cardo C. Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol 1995;147:545-559.
10. Couturier J, Sastre-Garau X, Schneider-Manoury S, Labib A, Orth G. Integration of human papillomavirus DNA near the myc genes in genital carcinomas and its consequence for proto-oncogene expression. J Virol 1991;65:4534-8.
11. Cross SM, Sanchez CA, Morgan CA, Schimke MK, Ramel S, Idzerda RL, Raskind WH, Reid BJ. A p53-dependent mouse spindle checkpoint. Science 1995;267:1353-1356.
12. Fu YS, Reagan JW, Richart RM. Definition of precursors. Gynecol Oncol 1981;12:220-31.
13. Holm R, Skomedal H, Helland A, Kristensen G, Borrensen A, Nesland JM. Immunohistochemical analysis of p53 protein overexpression in normal, premalignant, and malignant tissues of the cervix uteri. J Pathol 1993;169:21-6.
14. Jeffers MN, Richmond J, Farquharson M, McNicol AM. P53 immunoreactivity in cervical intraepithelial neoplasia and non-neoplastic cervical squamous epithelium. J Clin Pathol 1994;47(12):1073-76.
15. Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ. Human papilloma virus infection of the cervix; relative risk association of 15 common anogenital types. Obstet Gynecol 1992;79:328-37
16. Mourits MJE, Pieters WJLM, Hollema H, Burger MPM. Three-group metaphase as a morphological criterion of progressive cervical intraepithelial neoplasia. Am J Obstet Gynecol 1992;167:591-5.
17. Parmentier R, Dustin P. Reproduction experimentale d'une anomalie particulier de la metaphase des cellules malignes ("metaphase a trois groupes"). Caryologia 1951;1:99-109.
18. Pieters WJLM, Koudstraal J, Ploem-Zaayer JJ, Janssens J, Oosterhuis JW. The three-group metaphase as a morphologic indicator of high-ploidy cells in cervical intraepithelial neoplasia. Anal Quant Cytol Histol 1992;14:227-32.
19. Sacket DL, Haynes RB, Tugwell P. Clinical epidemiology: a basic science for clinical medicine. Boston/Toronto: Little, Brown and Company, 1985:59-138.

20. Scheffner M, Werness BA, Huibregtse JM, Levine JM, Howley PM. The E6 oncoprotein encoded by human papillomavirus type 16 and 18 promotes the degradation of p53. Cell 1990;63:1129-36.
21. Van Ranst M, kaplan JB, Burk RD. Phylogenetic classification of human papillomaviruses; correlation with clinical manifestation. J Gen Virol 1992;73:2653-60.
22. Winkler B, Crum CP, Fuji T, Ferenczy A, Boon M, Braun L, Lancaster WD, Richart RM. Koilocytotic lesions of the cervix. The relation of mitotic abnormalities to the presence of papillomavirus antigens and nuclear DNA content. Cancer 1984;53:1081-7.

Nederlandse samenvatting

Ondanks de dalende incidentie van baarmoederhalskanker (cervixcarcinoom) in de laatste 30 jaar blijft het cervixcarcinoom een relatief vaak voorkomende maligniteit die in Nederland jaarlijks ongeveer 300 doden eist. Cervixcarcinoom is een goed behandelbare ziekte indien deze in een vroeg stadium wordt ontdekt en behandeld. Via een uitstrijk verkregen cellen, het "uitstrijkje", kunnen zowel voorstadia van als kwaardaardige afwijkingen aan de baarmoederhals worden gevonden. Behandeling geschiedt in het algemeen door het verwijderen van het afwijkende weefsel.

Cervixcarcinoom wordt meestal vooraf gegaan door premaligne, nog niet infiltrerende afwijkingen welke cervical intraepitheliale neoplasieen (CIN) genoemd worden. CIN wordt onderverdeeld in 3 stadia, I t/m III. De kans op het ontwikkelen van een infilterend carcinoom neemt in het algemeen toe naar mate de CIN graad hoger is. Echter niet alle CIN leasies gaan over in infiltrerend carcinoom. Welke CIN laesies mogelijk in de toekomst kwaadaardig worden is op grond van de histomorfologie niet te voorspellen. Deze onzekerheid leidt tot het behandelen van patienten die waarschijnlijk nooit een carcinoom zullen ontwikkelen. Een ander nadeel van de CIN klassificatie is de substantiële intra-en interindividuele variatie die bij het graderen van CIN tussen pathologen bestaat.

In dit proefschrift is gezocht naar kwantitatieve kenmerken die de kans op het ontwikkelen van een infiltratief carcinoom uit CIN betrouwbaar zouden kunnen voorspellen. Tevens zijn factoren bestudeerd met een mogelijke etiologische rol in de genese van het cervixcarcinoom.

De onderzochte kenmerken zijn: de mitosen frequentie, het voorkomen van atypische mitosen, hoeveelheid DNA en infectie met het humane papillomavirus. Deze drie items zullen worden toegelicht en de bijbehorende hoofdstukken van dit proefschrift samengevat.

Mitose frequentie en atypische mitosen.

Maligniteit wordt onder andere gekenmerkt door verhoogd aantal mitosen en het voorkomen van atypische mitosen. In premaligne cervix afwijkingen is de beoordeling van mitosen van belang bij de gradering van deze laesies. Echter het feitelijk tellen en

onderverdelen van atypische mitosen heeft tot nu toe onder pathologen weinig aanhang gekregen. Er zijn verscheidene afwijkende verschijningsvormen van mitosen. In het oog springende afwijkende mitosen zijn de multipolaire mitoses, de mitosen met "lagging chromosomes", de "v"-vorm, en de "c"-vorm. Op deze atypische mitosen wordt ingegaan in dit proefschrift. Ons is gebleken dat mitosen met "lagging chromosomes", mn de driegroeps metafasen, gecorreleerd zijn met een verhoogde hoeveelheid DNA per kern, met de aanwezigheid van het humane papillomavirus (HPV) en voorkomen in CIN II en CIN III. Deze laatste bevinding vormde de basis voor de studies beschreven in hoofdstuk 2, 3 en 4.

In hoofdstuk 2 onderzochten wij een groep van 127 vrouwen met afwijkende uitstrijkjes (tenminste lichte tot matige dysplasie). Deze patienten ondergingen colposcopisch gerichte bioptering waarna excisie van de overgangszone plaatsvond. In het verwijderde weefsel werden volgens bepaalde richtlijnen mitosen geanalyseerd en geteld in het gebied met de hoogste aantal mitosen. De mate van proliferatie werd gedefinieerd als het aantal mitosen per 1000 kernen (mitotic index (MI)). Mitosen werden verdeeld in normale mitosen, multipolaire mitosen, mitosen met "lagging" chromosomen (lag type mitosen) en overige atypische mitosen. De lag type mitosen werden onderverdeeld in tweegroeps metafasen (2GM), driegroeps metafasen (3GM) en overige lag type mitosen (OLTM). We vonden dat de mediane MI waarde in de CIN I laesies 3 was, in de CIN II laesies 4 en in de CIN III laesies 9. De driegroeps metafase werd alleen in CIN II en CIN III leasies gevonden. Deze mitosevorm was sterk gecorreleerd met de aanwezigheid van de tweegroeps metafase en de overige lag type mitosen. De overige atypische mitosen bleken niet gecorreleerd te zijn met CIN stadium. Multipolaire mitosen waren zeldzaam. We concludeerden dat hogere CIN stadia meer lag type mitosen toonden en een hogere mitosenindex. Het voorkomen van overige atypische mitosen bleek statistisch niet gecorreleerd te zijn met het CIN stadium.

In hoofdstuk 3 werden mitosen geteld en geanalyseerd in 24 microinvasieve carcinomen met daaraan grenzende CIN III leasies en 82 cervix weefselstukjes zonder neoplastische kenmerken. We vonden dat de MI van de CIN leasies grenzend aan het carcinoom varieerde van 3 tot 17 met een mediane waarde van 7 terwijl de MI in het invasive carcinoom varieerde tussen 3 en 13 met een mediane waarde van 7,5. Zowel het invasieve carcinoom als de intraepitheliale deel toonden 2GM. 2GM kwam significant minder vaak voor in het carcinoom. Multipolaire mitosen waren zeldzaam terwijl nor-

male mitosen en overige atypische mitosen frequent voorkwamen.

De 82 niet neoplastische weefselstukjes waren afkomstig van twee groepen patienten: 31 patienten ondergingen een cervixbiopt ivm een afwijkend uitstrijkje (tenminste licht tot matige dysplasie) en bij 51 patienten werd de baarmoeder verwijderd voor niet cervix gerelateerde afwijkingen zoals baarmoederverzakking of vleesbomen. In 24% cq 26% van de weefselstukken werd een gebied met meer dan 2 mitosen per 1000 kernen gevonden waarin mitosen geanalyseerd werden. De geanalyseerde mitosen waren over het algemeen normale mitosen of overige atypische mitosen. Van de lag type mitosen werd de driegroeps metafase niet gevonden en de tweegroeps metafase werd in 37% (patienten met een afwijkende uitstrijk) cq 16% (patienten met niet cervix gerelateerde afwijkingen) gezien. We concludeerden dat de CIN laesie grenzend aan het microinvasief carcinoom gekarakteriseerd wordt door een MI met een minimum waarde van 3 and het voorkomen van tweegroeps metafasen. Deze combinatie werd, buiten 1 patient met basaalcelhyperplasie, niet gevonden bij de patientengroep zonder neoplastische afwijkingen aan de cervix. We concludeerden dat lag type mitosen maligniteit suggereren; ze zijn echter niet pathognomonisch voor maligniteit. Mitosenvormen anders dan lag type mitosen hebben geen diagnostische betekenis.

In hoofdstuk 4 werd de bruikbaarheid van het tellen en analyseren van mitosen getoetst. De twee onderzochte modellen voor de diagnose van cervical intraepitheliale neoplasieën waren de conventionel CIN classificatie met 3 stadia; het kwalitatieve model genoemd en het model gebaseerd op de analyse van mitosen, het kwantitatieve model genoemd. Volgens het kwantitatieve model was er sprake van een ernstige afwijking indien er een MI≥3 en een 2/3GM gevonden wordt.

In 225 patienten met een tenminste licht tot matige dysplastische uitstrijkje werden eerst colposcopisch, gerichte biopten genomen en later werd de gehele transformatie zone (deel van de baarmoederhals) verwijderd. Hierin werd de discrepantie tussen de op het biopt gestelde diagnose en de uiteindelijke diagnose in de twee modellen vergeleken. De uiteindelijk diagnose werd gedefinieerd als de meest ernstige afwijking gevonden in de biopten of het weefsel van de transformatiezone. We concludeerden dat het stellen van de diagnose met het kwantitatieve model in kleine laesies niet mogelijk was gezien het feit dat er niet voldoende mitosen geteld konden worden. Ook waren er minder discrepanties tussen biopsie diagnose en de uiteindelijke diagnose bij de conventionele CIN classificatie. Echter de helft van de CIN I en II laesies werden geclassificeerd

als ernstige afwijking volgens het kwantitiatieve model. Behandeling van deze patienten zou overwogen moeten worden.

Humane papillomavirus

Het humane papillomavirus (HPV) wordt in meer dan 90% van de cervixcarcinomen gevonden. Ook CIN leasies bevatten HPV waarbij in de hogere CIN klasses een hogere percentages. Het HPV speelt een belangrijke rol in de ontwikkeling van het cervixcarcinoom, echter de exacte pathogenese is nog niet duidelijk.

Er zijn inmiddels meer dan 60 HPV typen ontdekt. Bepaalde HPV soorten hebben meer affiniteit met de huid en andere weer met het slijmvlies van de tractus urogenitalis, waaronder de cervix. Van de HPV types die gevonden worden in de tractus urogenitalis blijken met name de types 16, 18 , 31 en 33 vaker bij cervixcarcinomen gevonden worden. Deze worden dan ook de oncogene HPV types genoemd. Andere typen, 6 en 11 worden gevonden in niet maligne genitale wratten. Deze types worden de benigne HPV types genoemd.

Humane papilloma viruseiwitten, het E6 en E7 eiwit, van de oncogene types interferen met de celcylcus en verstoren hiermee het fysiologische celgroei mechanisme. Het HPV E6-eiwit bindt aan het p53 eiwit. Het p53 eiwit vertraagt de aanvang van de celdeling (G1-arrest) in de celkern. Zodoende kan herstel van DNA fouten (mutaties) plaatsvinden. Mutaties kunnen leiden tot de vorming van maligniteit. P53 treedt tevens regulerend op bij de vorming van de kernspoel. De kernspoel is een structuur van glijdraden waarlangs de chromosomen tijdens de celdeling uiteengaan om twee identieke dochtercellen te vormen. Uit eerder onderzoek is gebleken dat verstoring van de functie van p53 leidt tot misvorming van de kernspoel en dat hierdoor mogelijk atypische mitosen ontstaan en een verhoogde hoeveelheid DNA. Verstoring van de celcyclus kan eveneens veroorzaakt worden door het E7 HPV eiwit dat bindt aan het retinoblastoma eiwit. Het retinoblastoma eitwit heeft evenals p53 eiwit een regulerende rol in de celcyclus. Een ander gen, c-myc gen, heeft evenseens invloed op de celcyclus. Het aflezen van dit gen kan verstoord worden doordat de inbouw van het HPV in het DNA vooral plaats vindt nabij het c-myc gen.

Hoofdstuk 5 van dit proefschrift beschrijft de relatie tussen mitosefrequentie en HPV type. Het risico voor het ontwikkelen van cervixcarinoom hangt mede af van het

type humane papillomavirus. Deze studie bevat 180 patienten met CIN III. HPV type werd gerelateerd aan MI en het voorkomen van lag type mitosen. We vonden dat HPV-16 geassocieerde leasies een significant hoger aantal lag type mitosen toonden dan HPV-18 of HPV-31. HPV-16 toonde de hoogste mediane waarde van de MI. HPV-16 en HPV-33 toonden een overeenkomstig aantal lag type mitosen. We concludeerden dat HPV infectie resulteerde in een verhoogd aantal celdelingen waarbij de verschillen in oncogene potentie tussen de verschillende HPV types mogelijk wordt vertaald in de hoeveelheid lag type mitosen in de CIN laesies. In hoofdstuk 6 wordt het aantal lag type mitosen (2GM en 3GM) gerelateerd aan de expressie van p53 in 74 patienten met CIN II of CIN III laesies. In deze serie konden we geen relatie tussen p53 expressie en het voorkomen van lag type mitosen aantonen. Evenmin was p53 expressie gecorreleerd met het HPV type.

DNA analyse

Al in 1902 stelde Boveri vast dat kanker veroorzaakt kan worden door "onevenwichtige" chromosomen paren. Inmiddels is bekend dat maligniteiten vaak een afwijkende aantal en/of afwijkende chromosoom vorm hebben. Ook bij premaligne afwijkingen blijken laesies met een afwijkende hoeveelheid DNA (aneuploid) per kern een ongunstiger verloop te hebben dan euploide (normale hoeveelheid DNA per kern) laesies. Vastgesteld kan worden dat aneuploide CIN laesies vaker progrediëren naar een hoger CIN graad of invasief carcinoom. Deze bevinding is gebruikt als basis voor hoofdstuk 7 en 8. In hoofdstuk 7 wordt het DNA gehalte vergeleken met HPV type in cervixuitstrijkjes van 345 patienten. Als maat voor een afwijkende hoeveelheid DNA werden cellen met een DNA gehalte van meer dan 5C gebruikt (normale cellen hebben een DNA gehalte van 2C). We vonden dat uitstrijkjes met HPV meer cellen met een DNA gehalte groter dan 5C bevatten. We concludeerden dat HPV infectie leidde tot een statistisch significant groter aantal cellen met een afwijkende DNA hoeveelheid vergeleken met de uitstrijkjes zonder HPV infectie. Deze bevatten in het algemeen een laag aantal cellen met afwijkende DNA gehalte.

In hoofstuk 8 beschrijven wij een analyse van de bruikbaarheid van DNA cytometrie voor de voorspelling van de histologische diagnose in 208 patienten met afwijkende cervix uitstrijkjes (tenminste licht tot matige dysplasie). DNA cytometrie is een

methode om het DNA gehalte van cellen te meten. Als maat voor afwijkende hoeveelheid DNA werd het aantal cellen met een DNA gehalte van meer dan 5C gebruikt (normale cellen hebben een DNA gehalte van 2C). Onder de 208 patienten toonden 110 patienten lichte tot matige dysplasie in de cervix uitstrijk en 98 patienten toonden ernstige dysplasie. In 94% van deze 98 patienten werd histologisch CIN II of CIN III gediagnostiseerd. De toegevoegde waarde van DNA cytometrie in deze groep patienten is gering. Bij de 110 patienten met licht tot matige dysplasie was de kans op een CIN II of hoger 54%. Met de introductie van DNA cytometrie kan de voorafkans op een CIN II of hoger vergroot worden. We vonden dat het aantal cellen met een DNA gehalte van meer dan 5C significant toenam met toenemend CIN stadium. Alle patienten met meer dan 52 cellen waarvan het DNA gehalte meer dan 5C bedroeg toonden een histologische diagnose van CIN II of meer. Echter maar 16(14%) van de patienten toonden 52 cellen met een DNA gehalte van meer dan 5C. Indien we een kans van 85% op een histologische diagnose van tenminste CIN II vereisten dan waren 27 cellen met een DNA gehalte van meer dan 5C nodig. Dit kwam voor bij 28(25%) van de patienten. We concluderen dat DNA cytometrie belangrijke informatie geeft gezien de significante relatie tussen het aantal cellen met een DNA gehalte van meer dan 5C en het CIN stadium. Echter, het aantal cellen met een DNA gehalte van meer dan 5C discrimineerde niet voldoende tussen de patienten met een CIN I en CIN II of erger. Daarom kan DNA cytometrie op zichzelf niet gebruikt worden als beleidsbepaler bij patienten met afwijkende uitstrijkjes.

In hoofdstuk 9 wordt een samenvatting gegeven van alle hoofdstukken en worden suggesties gedaan voor verder onderzoek.

Curriculum vitae

De schrijfster van dit proefschrift werd op 14 juli 1964 te Hazerswoude geboren. In 1981 behaalde zij het HAVO eindexamen en in 1983 haar Atheneum B examen aan het 's Adelbert College te Wassenaar. In 1983 werd begonnen met de studie geneeskunde die zij in 1991 cum laude afrondde met het artsexamen. In de wachttijd voor aanvang van de coschappen werd een wetenschappelijke stage doorlopen aan de University Hospital of Pennsylvania in Philadelphia. Tevens werkte zij gedurende haar studie als studentassistent bij de vakgroepen psychiatrie, huisartsgeneeskunde, gynecologie en verloskunde en pathologie.

In 1992 werd zij aangesteld als artsonderzoeker op het project tweetrapsscreening cervix dysplasieën olv dr. MPM Burger, dr. H Hollema en dr. WJLM Pieters waarvan de resultaten geleid hebben tot dit proefschrift.

In 1995 werd gestart met de opleiding tot patholoog aan het Academisch Ziekenhuis te Leiden (opleider: prof. dr. GJ Fleuren).

Nawoord

Een proefschrift schrijven doe je niet alleen!

dr. WJLM Pieters. Lieve Wim, in 1987 legde jij met jouw proefschrift over de drie groeps metafase de fundamenten voor dit proefschrift. Heel veel dank voor je inspirerende discussies, je luisterende oor en je gastvrijheid zowel thuis als op het laboratorium. Na de drie groeps metafase introduceerde je me in de pathologie; je toewijding aan het vak was al snel aanstekelijk. Zal ik ooit nog eens gaan tuinieren?

Lieve Diane, ook jou dank ik voor de gastvrijheid en je luisterende oor voor het verloop van dit project. Het is jammer dat de afstand Alphen-Sellingen frequent bezoek in de weg staat.

dr. H Hollema. Beste Harry, de laatste loodjes wegen het zwaarst. De laatste twee artikelen moesten uit mijn tenen komen maar uiteindelijk dankzij jouw inspirerende hulp kwamen ze er. Tijdens mijn "werkbezoekjes" aan Groningen wist je op een perfecte manier gezelligheid te combineren met "zaken doen". Ik zal die uitstapjes gaan missen!

prof. MPM Burger. Beste Matthé, dank voor je vasthoudendheid en het geloof in het slagen van dit project. Van jouw heb ik veel geleerd op het gebied van dataverwerking en artikelen schrijven. Ook wilde ik je bedanken voor de mogelijkheid die je me gaf om mijn werk met mijn gezin te kunnen combineren.

prof. J Aalders. Geachte professor, "het komt wel goed joh!" is een van uw statements en het kwam ook goed. Bedankt voor de inspirerende telefoongesprekken en het inzicht in "de rode draad van het verhaal".

prof. J Elema. Geachte professor, bedankt voor uw inzet en suggesties om dit proefschrift te vervolmaken.

Alle medewerkers van het laboratorium voor pathologie. Lieve allemaal, bedankt voor al die coupes die jullie voor me gesneden hebben, het zijn er uiteindelijk meer dan 3000 geworden. Maar nog dankbaarder ben ik voor jullie gastvrijheid op het laboratorium en bij enkele van jullie ook thuis. Ik voelde me al snel op mijn gemak in het oostelijke Groningen en uiteindelijk als een echte PA-medewerker, lid van de personeelsvereniging van het laboratorium voor pathologie.

drs. J Ploem-zaaijer. Lieve Joke, heel veel dank voor de invoering in het ingewikkelde proces van de LEYTAS. DNA-cytometrie heeft niet gebracht wat we ervan had

verwacht hadden. Toch blijft het een techniek die zeker in een andere onderzoekssetting zijn waarde zal tonen.

prof. GJ Fleuren. Beste Gert-Jan, het combineren van de opleiding pathologie en het schrijven van een proefschrift gaat niet altijd samen. Echter door de mogelijkheid om de opleiding pathologie in deeltijd te doen is dit boekje afgekomen. Bedankt voor je interesse en je participatie in de leescommissie.

prof. B Trimbos en prof. S Poppema dank ik voor hun participatie in de leescommissie.

Jaap, Vincent, Folkert, Hedda, Sandrine, Nathalie, Robert, Frans en Patty. Beste collega's hartelijk dank voor jullie interesse en morele steun.

Last but not least: Matthijs, in onze relatie is 1 + 1 meer dan 2. Dankzij jouw rotsvaste vertrouwen is dit ei gelegd.

Aan mijn dametjes, Maaike en Rozemarijn spreek ik de hoop uit dat uitspraken als "mama is werken" of "stil, want mama werkt" hopelijk in de toekomst wat minder frequent gehoord zullen worden.