Molecular Genetic Analysis of Cervical cancer

Using

Fluorescence In Situ Hybridization

and

Comparative Genomic Hybridization

by

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Abstract

The aim of this research was to investigate common cytogenetic changes in material from cervical tumours, using modern molecular techniques, in order to throw some light on the role of such changes in the initiation and development of cervical cancer, and to identify the potential locations of genes predisposing to this type of malignancy.

Cervical tumours nearly all have complex karyotypes, and more precise cytogenetic information is required to establish whether specific rearrangements occur, and if they are related to the type of HPV infection found. The karyotypes of five recently established cervical cancer cell lines, three from squamous cell carcinomas (two HPV 16 +ve and one HPV 18 +ve), one from an adenocarcinoma (HPV -ve), and one from an adeno-squamous carcinoma (HPV 16 +ve), have been analysed using fluorescence in situ hybridization (FISH), with 23 chromosome specific paints, YACs and cosmids as probes, in addition to conventional G banding.

Conventional G banding was used on metaphases from all 5 lines to obtain background karyotype information. A Fluorescence In Situ Hybridization (FISH) technique was applied to metaphases, using 23 different chromosome-specific paint probes to clarify the chromosome rearrangements. These techniques identified the origin of markers and revealed some common chromosome rearrangements.

The highest number of breakpoints occurred in chromosome 1. Breaks at 1q10/11 were seen in three different cell lines (DE3, JE6 and XH1) and a break at 1q21 occurred in all three squamous carcinoma lines. Isochromosomes i(1q) were found in two of the lines (DE3 & JE6). Double minutes seen initially in some cells from one squamous line, SM7, were shown also to originate from chromosome 1.

Chromosome 3 rearrangements were seen in all five cell lines, involving the short arm in the adenocarcinoma and the adeno-squamous carcinoma cell lines, and the long arm in all three squamous carcinoma cell lines. Yeast Artificial Chromosomes (YACs) and Cosmids, cloned
from chromosome 3, were used to map the precise breakpoints on this chromosome and all of them were found to be in different regions. An i(3q) was found in two lines out of five.

Small metacentrics involving chromosome 5 were a del(5q) in one line, and a t(X;5) in another, rather than the i(5p) observed by other investigators. The region 6q21 was involved in three cases, and chromosome 9 was rearranged in four. An i(8q) was found in the three squamous carcinoma cell lines. Structural changes of 11q were found in two cases, and a marker 11 representing amplification in the 11q14-22 region was duplicated in the adenosquamous line.

The Comparative Genomic Hybridization (CGH) technique was applied to three of the cell lines, the squamous line, SM7, the adenocarcinoma line, JE6, and the adeno-squamous line, XH1. All these lines were part of the previous investigation, so it was possible to test the sensitivity of CGH on cell lines where all the major cytogenetic changes had been catalogued in detail.

For the near-tetraploid squamous carcinoma cell line, SM7, the hybridization and CGH profiles reflected clearly the numbers of each chromosome present, and the breakpoints of unbalanced aberrations. For the hyper-diploid adenocarcinoma line, JE6, most of the chromosomes present as 2 copies had a profile of 1, but several did show lower profiles which did not represent losses. Most extra chromosomes and regions of change could be clearly seen and, as expected, the reciprocal translocation was not detectable. Hybridization was not of such good quality as the other two, for the tetraploid adeno-squamous line, XH1, and it was not possible to tell which whole chromosomes were under or over-represented. Where there were changes in copy number along the length of the chromosome, however, the shift in ratio profile was nearly always in the right direction, giving some indication of the position of the breakpoints. A region of amplification (11q14-22) showed up very clearly, as well as a deletion at 11q23, not observed by FISH. This could be one of the potential tumour suppressor loci involved in cervical cancer.
From the CGH profiles for the fresh primary tumour DNA, it was possible to observe obvious profile changes not associated with centromeres and telomeres, which could indicate breakpoint sites. When these were compared with breakpoints in the squamous carcinoma cell lines, 3q10 and 8q10 were common to both, indicating that over-representation of 3q and 8q seen in cell lines was also present in an uncultured tumour, and therefore could be important cytogenetic changes in vivo.

In summary, karyotyping of cervical cancer cell lines using G-banding and FISH has shown frequent involvement of chromosomes 1, 3, 6, 8 and 9. Comparisons between CGH and conventional karyotyping have shown that breakpoints, as well as amplifications and deletions, can be mapped by CGH. Over representation of all or part of 3q and 8q in material from four squamous tumours by both techniques shows that these are consistent genetic changes which could provide useful markers for invasive squamous carcinoma of the cervix.
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**Abbreviations**

A  adenine or adenosine
bp  base-pairs
BSA  bovine serum albumin
BSS  balanced salt solution
C  cytosine or cytidine
C-banding  constitutive heterochromatin banding
CCD  charge coupled device
cDNA  complementary DNA
CGH  comparative genomic hybridization
cM  centimorgan
DAPI  4, 6-diamidino-2-phenyl-indole
dDH2O  deionised and distilled water
DM  double minute
DMEM  Dulbecco's modified Eagles medium
DMSO  dimethysulphoxide
DNA  deoxyribonucleic acid
EDTA  ethylenediamine tetraacetic acid
FCS  foetal calf serum
FISH  fluorescence in situ hybridization
FITC  Fluorescein isothiocyanate
g  grams
G  guanine or guanosine
G-banding  Giemsa banding
GTP  guanosine triphosphate
HPV  human papilloma virus
HSR  homogeneously stained region
HSV  herpes simplex virus
kb  kilobase-pairs
kDa  kilo Dalton  
L  litre  
LOH  loss of heterozygosity  
M  moles  
Mb  megabase-pairs  
MW  molecular weight  
PBS  phosphate-buffered saline  
PCR  polymerase chain reaction  
PHA  phytohaemagglutinin  
pM  picomolar  
pmol  picomole  
RFLP  restriction fragment length polymorphism  
RNA  ribonucleic acid  
RNase  ribonuclease  
SSC  saline sodium citrate  
SV40  Simian virus 40  
T  thymidine or thymine  
Tris  tris(hydroxymethyl)aminomethane  
TRITC  tetramethyl rhodamine isothiocyanate  
\(u\)  units  
\(\mu g\)  microgram  
\(\mu l\)  microlitre  
\(\mu M\)  micromolar  
\(v/v\)  volume for volume  
\(w/v\)  weight for volume  
YACs  yeast artificial chromosomes
Chapter 1

Introduction
Chapter 1.

Introduction

This investigation was concerned with the molecular cytogenetic aspects of cervical cancer. It involved a detailed analysis of the karyotypes of cervical cancer cell lines using G banding, Fluorescence In Situ Hybridization (FISH), Comparative Genomic Hybridization (CGH), and further application of CGH to DNA from fresh tumour material was also used.

1.1. Cervical Cancer

1.1.1. Definition and incidence

Cervical cancer is a carcinoma of the neck of the womb or cervix, and by virtue of its accessibility, it can be diagnosed in its pre-invasive state.

In the U.K. in 1988, nearly 5000 new cases of cervical cancer were registered, making it the eighth commonest cancer in women, with an annual incidence rate of 169 new cases per million population (CRC Factsheets 12.1. and 1.3. 1994).

Nearly half a million new cases of cervical cancer occur annually worldwide, and in 1985 it comprised 6% of all the cancer diagnosed. Almost 80% of the new cases of cervical cancer occurred in developing countries, where it was the third most common form of cancer (CRC Factsheet 22.1. 1995).

Cervical cancer currently claims the lives of nearly 1600 women in the UK every year, making the mortality rate 52 per million population, the 12th commonest cause of cancer death in women in the UK in 1994 (CRC Factsheet 3.3. 1995).

Over the last twenty years, UK mortality rates from cervical cancer have decreased substantially from 88 per million population in 1972 to 63 per million population in 1992. However, the incidence rates have changed little (CRC Factsheet 12.2. 1994).
1.1.2. Pathology

Carcinoma of the cervix may develop in either the squamous epithelium or the glandular epithelium of the endocervix. Approximately 90% of the growths are squamous carcinomas and the remaining 10% adenocarcinomas. Histological grading varies from poorly differentiated to well differentiated.

Invasive cancers of the cervix are either exophytic, hypertrophied and producing cauliflower-like growths or eroding and ulcerative. The tumour spreads to nearby structures such as the body of the uterus, vagina, bladder parametrial tissues, broad ligament and uterosacral ligaments by direct extension. Lymphatic spread may occur early in the disease and the nodes commonly involved are the obturator, external iliac, internal iliac, common iliac, sacral and ultimately the para-aortic. Spread via the blood circulation is much less frequent, but embolic metastases are occasionally seen in the ovary, brain, bone and lung (Peel, 1986).

1.1.3. Clinical features and Treatment

The most common presenting symptoms are post-coital and post menopausal bleeding. Cervical cancer diagnosis is basically a clinical one confirmed by histology of the biopsied specimen. Since the success of the chosen regimen very much depends on the staging, thorough pre-treatment assessment and staging is important and essential. Surgical treatment is for patients whose disease is within the FIGO staging la, 1b and IIa. It is of the greatest advantage to the younger woman, where it can offer the preservation of ovarian function. When the disease has spread into the parametrium, radiotherapy is more suitable. Chemotherapy has been used for advanced disease (Peel, 1986).
1.1.4. FIGO Staging for cervical cancer
(The Federation Internationale de Gynécologie et Obstétriques)

The definition of the clinical stages in cervical cancer remain the same as agreed in 1985. At that time, the definition of micro-invasion was altered and the term 'occult' abandoned.

Current staging is as follows:

Stage I The carcinoma is strictly confined to the cervix

Ia Pre-clinical carcinoma of the cervix, those cases diagnosed by microscopy only

Ia1 Minimal microscopic stromal invasion

Ia2 Lesions microscopically measured; <5mm in depth from base of epithelium and <7mm in horizontal measurement.

Ib Lesions of greater dimension than stage Ia2 whether clinically seen or not

Ib1 Clinical lesion not greater than 4.0 cm

Ib2 Clinical lesion greater than 4.0 cm
Stage II
The carcinoma extends beyond the cervix, but has not extended on to the pelvic side wall or the lower third of the vagina.

IIa Without obvious parametrial involvement

IIb With parametrial involvement

Stage III
The carcinoma has extended on to the pelvic side wall, and/or the tumour involves the lower third of the vagina and/or hydronephrosis or non-functioning kidney

111a No extension on to the pelvic side wall, but involvement of the lower third of the vagina

111b Extension on to the pelvic side wall, and/or hydronephrosis or non-functioning kidney

Stage IV
The carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or the rectum.

1Va Spread of the growth to adjacent organs

1Vb Spread to distant organs
### 1.1.5. Prognosis

Important factors indicating a poor prognosis in cervical cancer are: advanced stage, presence of pelvic or para-aortic lymph node involvement, adenocarcinoma rather than squamous carcinoma, poorly differentiated tumour and the presence of lymphatic or vascular permeation by the tumour (Peel, 1986).

When pelvic lymph nodes are affected by the tumour the 5 year survival falls to 50%. The 5 year survival rates in the different FIGO stages are as follows:

<table>
<thead>
<tr>
<th>FIGO Stage</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>80-85%</td>
</tr>
<tr>
<td>II</td>
<td>60-70%</td>
</tr>
<tr>
<td>III</td>
<td>30-40%</td>
</tr>
<tr>
<td>IV</td>
<td>&lt;15%</td>
</tr>
</tbody>
</table>
1.1.6. Normal and abnormal histology and cytology of cervical epithelium

Normal histological features of cervical squamous epithelium

The squamous epithelium covers the ectocervix and continues on to the vaginal epithelium. It is stratified, non-keratinised and separated from the underlying stroma by a basement membrane. Differentiation, maturation and stratification are used in describing normal and abnormal epithelium (Figure 1 & 2).

Differentiation

This refers to the process whereby the squamous cell becomes flattened and functions as a protective layer. In the normal cervical epithelium, keratinization is not involved in differentiation. Cervical cells differentiate as they mature.

In abnormal cervical epithelium, differentiation relates to the degree of morphological and functional similarity between abnormal and normal at all stages of maturation. Differentiation is disproportionate to maturation when the epithelium becomes more abnormal.

Maturation

Maturation is closely related to differentiation and mature cells show good differentiation. Maturation is seen when cells reach the surface of normal squamous epithelium.

Stratification

Epithelium is divided into layers of progressively more mature and flattened cells as the surface is reached, and there is a compulsory sequence of maturation and differentiation.

The mature squamous epithelium of the ectocervix has a well defined basal layer attached to the basal lamina. The next layer is the parabasal cells and both of these layers have a high nuclear : cytoplasmic ratio. Cells in the intermediate zone are slightly flattened with a moderate sized round nucleus. The nucleus becomes smaller and the cytoplasm became more abundant as maturation progresses towards the surface. The superficial layer has markedly flattened cells, eosinophilic cytoplasm and small flattened pyknotic and dense nuclei (Anderson et al., 1992).
Figure 1  Diagrammatic representation of cervical squamous epithelium
a. histology  b. cytology

Figure 2  Normal histological features of cervical squamous epithelium
a. Whole thickness showing a well defined basal layer attached to the basal lamina
Pictures showing differentiation and maturation

d. Intermediate and superficial layers

e. Basal and parabasal layers
Normal cytological features

The cytological preparations are stained by the standard Papanicolaou method. Haematoxylin is used to stain the nucleus (Figure 3).

Parabasal squamous cells

They have a round shape with well defined borders, a well defined, darkly stained, coarse chromatin pattern, and a large nucleus which occupying 80-90% of the cell. The cytoplasm appears thick and is of a homogeneous blue colour.

Intermediate cells

In these cells the amount of cytoplasm is considerable and the nucleus is small. The cytoplasm is basophilic but thin and transparent, and a well defined yellow colour region is found around the nucleus because of the presence of glycogen. The nucleus is round with a fine pale granular chromatin structure.

Superficial squamous cells

These cells are large and flattened, with angled edges of a polyhedral shape. The cytoplasm is transparent and pink in colour. Nuclei are small and dense, without a chromatin structure.

Endocervical cells

Collection of glandular cells of endocervical origin usually appear in groups or sheets. Nuclei are basophilic, darkly stained, with a prominent fine chromatin structure, basally located in cylindrical columnar cells. The cytoplasm is pale blue with poorly defined cell borders. The cells are approximately five times the size of mature squamous cells (Anderson et al., 1992).
Figure 3 Two examples of normal cervical smears

a. Superficial squamous cells at mid cycle

b. Mixture of superficial, intermediate squamous cells and endocervical glandular cells
Abnormal histological features

Cervical Intraepithelial Neoplasia

(Figure 4)

CIN I

Good maturation with a minimal degree of nuclear abnormalities, mainly confined to the deeper layers of epithelium.

CIN II

Maturation is present in the upper half of the epithelium. Nuclear abnormalities are more marked than CIN I and extend towards the superficial layers. Abnormal mitotic figures are seen in the basal half of the epithelium.

CIN III

Differentiation and stratification may be completely absent or only present in the superficial layer. Nuclear abnormalities may extend throughout the thickness of epithelium to a more marked degree than CIN I and II. Mitosis may be abundant and abnormal forms are frequent.

Abnormal cytological features

(Figure 5)

The distribution of cells in a cervical cytology smear bear no relationship to each other. Cytological assessment is carried out on individual cells in order to identify dyskaryosis and assess the degree of dyskaryosis.

Cytological features of dyskaryosis include nuclear and cytoplasmic changes. Nuclear changes involve nuclear enlargement, variation in size and shape, hyperchromasia, abnormal appearance and chromatin distribution, irregular nuclear outline, irregular thickening of the nuclear membrane, and the presence or absence of nucleoli and mitotic figures (Anderson et al., 1992).
Figure 4  Abnormal histological features of cervical epithelium

a. CIN I

b. CIN II

c. CIN III
Figure 5  Abnormal cytological features of cervical epithelium

a. Mild dyskaryosis

b. Moderate dyskaryosis

c. Severe dyskaryosis
Cytological changes induced by Human Papilloma Virus (HPV)

Papilloma viruses are members of the Papovaviruses. They are DNA viruses. The virion is 52-54 nm in diameter. They are heat resistant and ether stable. Papovavirus causes warts in man in various morphological forms on the hands, feet, larynx and genitalia (Cruickshank et al., 1980).

Cytological features associated with HPV infection are koilocytic change, binucleation and individual cell keratinization (Figure 6 a 7 b). In general, a koilocyte is regarded as a pathognomonic feature of HPV infection. However, koilocytes are only identified in 50 - 70% of samples with HPV infection. The koilocytes are cells with enlarged nuclei with irregular nuclear chromatin surrounded by large irregular shaped halos. The margin of such a halo shows cytoplasmic condensation with a well defined edge (Figure 6 c) (Anderson et al., 1992).

The degree of nuclear changes associated with HPV infection could be difficult to differentiate from CIN, and such changes may be mistaken for CIN. In these circumstances the smear could be reported as 'borderline change', and interpretation could give rise to either over or under diagnosis. However, application of criteria and an accurate assessment can make the distinction between cytological changes due to HPV infection and CIN possible. HPV infection can cause increased mitosis in the cervical epithelium, but these should not be mistaken for CIN if other features of HPV are present. Nevertheless, sometimes CIN and HPV infection can co-exist in the same area of epithelium (Anderson et al., 1992).
Figure 6  Histological and cytological features of HPV

a. Koilocytic change

b. Individual cell keratinization and multinucleation

c. HPV infection seen on cervical smear
In 1941, Papanicolaou suggested that the examination of smears would allow pre-invasive carcinoma of the cervix to be recognised in the asymptomatic population, and that treatment of the disease at this stage would prevent the onset of cervical cancer and reduce the incidence of mortality from this disease. Papanicolaou gave his name to the 'Pap' smear and laid the foundation for preventive medicine many years before national screening programmes developed (Peel, 1986).

In 1951 Ayre improved the precision of the test by designing a special shaped wooden spatula to collect cells from the squamocolumnar junction of the cervix. The cells were spread onto a glass slide and fixed with 95% ethanol. Slides were examined after staining by the Papanicolaou stain or with haematoxylin eosin stain. At that time, cytologists recognised smears with cells undergoing inflammatory changes without any evidence of malignancy, some smears with cells suggesting dysplasia reported as 'doubtful', and positive smears, which included cells suggestive of severe dysplasia, and carcinoma-in-situ or invasion. This classification did not represent a definite diagnosis (Peel, 1986).

In 1975 the World Health Organization recommended a distinction of the pathological features of the various stages of the disease and cervical intraepithelial neoplasia has gradually replaced the older terms of dysplasia and carcinoma-in-situ (Peel, 1986).

The concept of cervical intraepithelial neoplasia included the notion that all degrees of abnormality should be given the same name as part of a continuous spectrum of one disease. CIN I is equivalent to mild dysplasia, CIN II to moderate dysplasia and CIN III includes both severe dysplasia and carcinoma-in-situ. The advantages of this system are that the CIN classification can recognise even the mildest form of one end of the CIN spectrum. It is compatible with the natural history of the disease and also consistent with a modern
management approach. The disadvantage is that sometimes very minor, often reversible changes have been labelled as CIN, leading to over diagnosis and over treatment (Anderson et al., 1992).

Cytological criteria must be based on individual cell changes. Therefore 'dyskaryosis' is preferable to CIN which is used by histopathologists after examining the whole tissue and the relationships between cells. Dyskaryosis is also divided into three grades, mild, moderate and severe and these are aligned closely to the histological grades, as shown in the table (Anderson et al., 1992).
The terminology is summarised in the following table:

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>CIN I</th>
<th>CIN II</th>
<th>CIN III</th>
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</thead>
<tbody>
<tr>
<td><strong>Histological</strong></td>
<td>Benign lesions</td>
<td>Squamous metaplasia</td>
<td>Mild dysplasia</td>
<td>Moderate dysplasia</td>
</tr>
<tr>
<td><strong>diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytological</strong></td>
<td>Normal or Inflammatory</td>
<td>Squamous metaplasia</td>
<td>Mild dyskaryosis</td>
<td>Moderate dyskaryosis</td>
</tr>
<tr>
<td><strong>diagnosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Papanicola</strong></td>
<td>Class 1 + 2</td>
<td>Class 3</td>
<td>Class 4</td>
<td>Class 5</td>
</tr>
<tr>
<td><strong>-ou</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DOH</strong></td>
<td>Class 2</td>
<td>Class 3</td>
<td>not classified</td>
<td></td>
</tr>
<tr>
<td><strong>DOH</strong></td>
<td></td>
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DOH = Department of Health

Anderson et al., 1992

(A Text and Atlas of Integrated Colposcopy)
1.1.8. Natural history of cervical cancer

In general it has been shown that a mild or moderate degree of CIN will either progress to a more severe form or it will regress. Regression is more likely in the milder forms. Although there is no evidence, it was assumed that progression through the grades of CIN occurs in a gradual and incremental fashion. However, severe grades of CIN may develop without necessarily being preceded by low grade minor abnormalities. The possibility is that CIN represents abnormal clones of cells and a clone with a severe grade may develop within a field of minor abnormality (Anderson et al., 1992).

Cervical screening programmes which detect CIN and allow treatment of the pre-invasive phase were based on some assumptions; firstly that a significant proportion of women with CIN would eventually develop invasive tumour if not treated, and secondly that most invasive carcinomas are preceded by a demonstrable CIN phase (Anderson et al., 1992).

A prospective study performed by Lie et al., 1995 (APMIS) has shown that about 47% of low grade CIN lesions will regress, 37% will persist and 16% will progress to a high grade CIN. Ten years before that, McInndoe W A et al. (1984), in a prospective follow-up study, showed that 22% of CIN III cases progress to invasive cancer.

However, there is little precise information on the rate of progression from CIN to invasion because of the ethical impossibility of observing women with a known pre-malignant disease without intervention. In the 1950's, two studies from Scandinavia showed that 65-70% of women with CIN III, developed invasive carcinoma over the course of 12 years. A recent study from New Zealand found that 18% of women who had persistent abnormal cytology following incomplete treatment for CIN III developed invasive carcinoma after 10 years and 36% after 20 years. However, those patients in the New Zealand study were partially treated, hence this study did not represent the natural course of the disease. This demonstrates the main flaw in this type of study, which is the inability to make a reliable diagnosis without removing tissue. This could interfere with the natural progression of the disease (Anderson et al., 1992).
The epidemiological and cytological studies indicate that cervical CIN takes at least 10 years to become invasive, although more recent data would suggest that it may be less (Anderson et al., 1992).

Some young women have developed invasive carcinoma of the cervix following recent negative cytology. If we exclude the patients with genuine false negative smears, it appears that at least in some women there was a genuine progression from normal cervix to invasion in a short time, in months rather than years. Likewise, another small group of women fall at the other end of the distribution curve of the disease natural history, and progression to invasion may never occur in the course of a lifetime. Because of the moral and ethical barriers to necessary studies, it seems unlikely that precise data for the rate of progression from CIN to invasion will ever be known (Anderson et al., 1992).
1.1.9. Screening

The aim of cervical cancer screening is to reduce mortality from cervical cancer by regularly screening all women at risk in order to identify and treat conditions that might otherwise develop into cancer (CRC Factsheet 13.1. 1994).

Screening programmes have been in operation in Europe and North America for over 20 years and were started in the UK in 1967. In the past, screening in the UK was not effective, because it failed to reach the women who were at risk. Before 1988, two thirds of the patients with cervical cancer and 90% of the over 40 age group had never been screened. However, the situation has improved in recent years. In 1991-2, with a total of over 45 million smears being examined (screening, diagnostic and follow up smears) and coverage of the target population increased from 43% in 1988-9 to 83% in 1992-3 in women between the age of 20-64 in England and 15-59 in Scotland (CRC Factsheet 13.4 1994).

An apparent increase in the incidence of CIN in women under 35 is partly due to the screening programme, but also represents a true rise. To be more effective, screening has been offered to a wider range of people, including older women of low socio-economic class who were previously unscreened. Coverage of the target age range is a more important determinant of risk reduction than the frequency of screening within the defined age range (CRC Factsheet 13.5. 1994).

If a woman is screened regularly every 5 years from age 20-64, her risk of developing cervical cancer is reduced by 84%. Decreasing the interval to 3 years, adds 7% extra protection, with an increase in the number of smears in her life time from 9 to 15. Decreasing the interval still further to 1 year, adds an additional 2 % protection, increasing the number of smears to 45. In the UK in 1991, 46% of health authorities operated a 5-year recall, 39% a 3-year recall and the remaining operated a mixture of 3-5-year recall according to age.

Screening programmes of this kind are clearly effective, but enormously expensive as it includes large numbers of patients, who would otherwise regress naturally, are treated.
Clearly, the availability of a simple test which could distinguish between those CINs more likely to develop into invasive cancer and the remainder would be an enormous advantage.

1.1.10. Epidemiology

The risk of developing cervical cancer is closely related to sexual habits and promiscuity. Cervical cancer does not occur in women who have never had sexual intercourse, and coitus is the major prerequisite for the development of this tumour. Early age of first intercourse is important, but the number of sexual partners is the most important factor, and this appears to be independent of the age at first intercourse. The risk for a woman is also related to the number of sexual contacts her partner has had. The disease is known to be rare in nuns. Oral contraceptive use has been associated with an increased risk, but this may be a reflection of sexual behaviour (Peel, 1986; Anderson et al., 1992).

In general the disease is commoner in women of lower socioeconomic status. It is associated with poverty, and it is a major cause of death in developing countries.

The occupation of partners has been examined as a possible risk factor in view of the association with promiscuity and the possible exposure to carcinogens such as tar or oils. High incidence rates of cervical cancer have been associated with similar high rates of penile cancer, but, there has been no support for the idea that male circumcision may protect from cervical cancer. Smoking is thought to increase susceptibility to the disease (Peel, 1986).

Because of the importance of sexual behaviour in the aetiology of cervical cancer, sexually transmitted infection such as Human Papilloma virus and Herpes simplex virus type II have been studied as possible aetiologic factors.
1.1.11. Viruses associated with cervical cancer

Human Papilloma Virus

In the last 20 years human papilloma virus (HPV) has been at the centre of investigations in relation to the aetiology of cervical cancer. The prevalence of HPV DNA in cervical cancer samples and high grade CINs has been in the range of 85 - 100% by PCR and/or high quality Southern hybridization (Second International Workshop on The Epidemiology of Cervical Cancer and Human Papilloma Virus, 1991, published 1992).

F.X.Bosch (Barcelona) and M.M.Manos (Baltimore) set up a multinational collaborative project and collected over 1000 invasive cervical cancer samples from 32 hospitals in 22 countries across the world from 1989 to 1992. Their aim was to determine the association between HPV infection and cervical cancer and investigate geographical variation in the distribution of HPV types. The outcome of this International Biology Study on Cervical Cancer (IBSCC) was published in 1995 in the Journal of the National Cancer Institute.

Bosch, Manos and co-workers found over 25 different HPV types in their study, and HPV DNA was detected in 93% of the tumour samples collected. HPV 16 was found in 50% of the specimens, HPV18 in 14%, HPV 45 in 8% and HPV 31 in 5%. Geographically, HPV45 was more common in Western Africa, HPV 39 and 59 in Central and South America, and HPV 18 in Indonesia. Histologically, HPV 16 was found predominantly in squamous cell carcinomas (51%), and HPV 18 was found in 56% of adenocarcinomas and 39% of adeno-squamous carcinomas.

Recently published in 1996, a Danish case-control study from 1991 to 1993, reported by S K Kjaer and co-workers, has shown that the relationship with HPV was observed in all grades of CINs and the strength of association was greater in high grade CINs. The association between HPV and CIN was pronounced in oncogenic HPV types, HPV 16,18, 31 and 33. The overall prevalence of HPV in all grades of CIN was 66%, but it was 80% in histologically confirmed high grade CINs. Kjaer et al. also found that the HPV X group was a heterogeneous group with the possibility that it contained unidentified high risk types (Meijer et al., 1994).
Ley et al., 1991 and Melkert et al., 1993 have shown that 20 - 40 % of sexually active and cytologically normal young women have detectable HPV infection. From previous studies, Bosch and Manos have made the observation that HPV 16 was commonly found in women with normal cytology, CIN and cervical cancer.

HPV infection of the cervix can be divided into two groups: types associated with low grade CIN and rarely with invasive tumours such as HPV 6, 11, 31 & 35, and those associated with high grade CIN and progression to invasion such as HPV 16 & 18. Viruses in this second group have the ability to transform cells in culture and to cooperate with oncogenes in cellular transformation. When the cell is infected by HPV, the virus persists in the nucleus as circular DNA, known as 'episomal' which is separate from the cellular DNA. However, in cervical tumours, HPV DNA is integrated within the host genome instead of being episomal (Anderson et al., 1992; Peel, 1986).

Bernard et al. (1994) identified over 35 HPV types in female genital tracts by PCR and RFLP techniques, and nearly 70 different HPV types were reported by Richart & Wright, 1992. However, some of them are non-oncogenic. Meijer, (1994), unpublished data, has shown that HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 54, 56, 58, 61 and 66 are oncogenic and 6, 11, 13, 32 and 40 are non-oncogenic. HPV Xc, d, f, g were renamed as HPV 54, 61, 55 and 40 respectively. In their study, HPV X was positive in 12% of Pap IIIa (mild) smears, in 8% of Pap IIIb (moderate) smears and in 2% of Pap IV (severe) smears.

In 1995, Dyer Montgomery et al. and Popescu et al. showed that HPV transfection can immortalise human cervical cells, but that additional involvement of certain oncogenes is necessary to make these cells tumourigenic. In the same year, Zimonjic et al. demonstrated the same molecular genetic outcome in HPV negative cervical carcinomas. The nature of HPV negative cervical cancer is still a subject of much discussion. There are three possible hypotheses: either HPV negative cervical cancer is a different biological entity, the aetiology of which is not related to HPV, or HPV DNA disappears during the evolution of the tumourigenic process, or there may be some limitation of detection, or a sampling error or primer sensitivity.
**Herpes Simplex Virus type II**

Patients with cervical cancer have a greater prevalence of HSV 2 antibodies than women without cancer, but HSV 2 DNA has only rarely been found in cervical tumour samples. It is highly likely that the prevalence of HSV 2 antibodies is more closely related to sexual behaviour than to the presence of neoplasia (Anderson *et al.*, 1992).

Richart and Wright, (1992) have also observed that transcriptionally active HSV DNA is rarely seen in cervical cancer and the proteins encoded by HSV genes are not always found. Therefore they do not regard HSV 2 as an aetiological agent of cervical cancer.

Although HSV 2 is capable of transforming cells in culture, laboratory studies have not provided clear molecular, immunological or experimental evidence to support the role of HSV-2 in cervical carcinogenesis. Recent epidemiological studies have not clarified this uncertainty because the number of cases in these studies were too small to allow any definite conclusion to be reached (Nair and Pillai, 1992)(Review).
1.2. Cancer and Genetics

1.2.1. Introduction

One of the important developments in cancer research in the last decade has been the provision of evidence to support the hypothesis that cancer is in essence a genetic disease at the somatic cell level. It has been shown that cancer is a multi-step process arising from the accumulation of several different mutations (Vogelstein and Kinzler, 1993)(Review). The progression of a tumour from a normal cell results from a series of somatically acquired genetic changes (Rabbitts, 1994)(Review).

At the molecular level, being able to identify the somatic alteration of several genes in sequence in relation to different stages of cancer has also supported the multi-hit theory of carcinogenesis. Phenotypes depend on which altered loci have accumulated, the nature of the mutation at each locus, and the order in which these changes have occurred (Klinger, 1991).

The discovery of oncogenes and tumour suppressor genes has shown that cancer is not only a genetic disease at the somatic level, but at the germ-line level as well (Pierotti et al., 1992).

1.2.2. Chromosome rearrangements in cancer

In the 1970s, with the advent of banding techniques, it became possible to identify each chromosome individually and begin to recognise specific chromosome changes in different tumour types. It was also possible to characterise a large number of non-random chromosome changes associated with both haematological and solid tumours (Nowell, 1992).

Consistent and specific chromosome changes have been discovered in an increasing number of tumour types, providing convincing evidence of the fundamental role of chromosome rearrangement in carcinogenesis (Nair et al., 1992)(Review).
There are three types of chromosome rearrangements in cancer:

Type I: primary abnormalities which are essential in initiating and establishing the neoplastic process. They are found in the early phases of tumourigenesis. These changes correlate with the tumour type.

Type II: Changes which are strongly associated with a particular cancer, but may also occur in other types. They occur later and are important in tumour progression. These abnormalities result from genetic instability of the tumour which predisposes to further mutations and leads to genetic and phenotypic variability within the tumour cell population.

Type III: Random changes that are referred to as cytogenetic noise or background nonsequential abnormalities (Nair et al., 1992)(Review).

Both benign and malignant tumours have demonstrable karyotypic alterations. Clinically advanced tumours have more karyotypic rearrangements. Additional chromosomal changes in relation to clinical progression have shown that tumour progression results from additional somatic genetic alterations (Nowell, 1992).

Chromosomal abnormalities in human tumours have provided the clues to the locations of growth regulatory genes involved in oncogenesis. They have also indicated the mechanisms by which the function of these genes has been altered, resulting in cancer (Nowell, 1992).
1.2.3. Oncogenes

Since the 1960's, viral genes that directly transform normal into tumour cells have been studied widely. There are two proposed pathogenic mechanisms of virus inducing neoplasia. The first mechanism concerns persistent viral genomes in tumour cells. At some stage the tumour cell ancestry must have been infected by the tumour inducing virus. The virus carries a gene which initiates and maintains neoplasia. The second mechanism concerns suppression of the host immune system. The tumour cell ancestry need not be infected by the virus, but as an indirect result following infection of other cells, immune suppression results, impairing elimination of tumour cells, and therefore increasing the targets for other carcinogenic changes (Wyke, 1986).

The viruses that transform cells in culture and induce tumours in animals are the chicken and mouse retroviruses and the papovaviruses. Transforming genes in these viruses encode proteins which initiate and/or maintain abnormal cell growth. The transforming genes are of two groups. The first group consists of transforming genes from retroviruses which play no part in viral replication. These genes are related to sequences in normal host cells from which they are believed to have evolved after capture of the cell gene by the virus in some ancestral infection. They are known as viral oncogenes (v-oncs). Their cellular counterparts are known as cellular oncogenes or protooncogenes (c-oncs). The second group of transforming genes are those in polyoma and SV40 viruses. They play a part in the virus life cycle and are not derived from cellular ancestors (Wyke, 1986).

Each viral oncogene (v-onc gene) is given a name, a three letter word to define the virus from which it was isolated. For example, src onc gene was from the retrovirus, Rous sarcoma virus of chicken. Cellular and viral src genes have almost identical nucleotide sequences, with only two exceptions. The v-src has no introns. The last carboxy terminal 12 coding aminoacids of v-src are different from that of c-src. The cellular DNA contains genes which when transplanted or transduced into retroviruses, are cancer causing genes. However, cellular genes cannot operate as cancer genes in their own cells. The cellular onc genes may be activated and over-expressed in some tumours due to chromosome
translocation which may alter their regulation because of their new position, gene amplification or mutation (Teich, 1986).

In addition to v-src and c-src, abl, fes, fgr & fms, a large number of viral and cellular oncogenes have been isolated. Each v-onc shows the transduction of a separate cellular gene, on the basis of observation of their cellular homologues mapped to different chromosomes. They all have a small region of homology resulting in similar kinase function. The oncogene erbB shares this sequence. Two others, raf and mos, also show kinase activity. These oncogenes with shared biochemical functions are known as the protein kinase family (Teich, 1986).

The ras oncogenes were originally discovered in Harvey and Kirsten murine sarcoma viruses. They were not homologues and were found at two separate chromosome loci. N-ras, showing homology to Ha-ras and Ki-ras, was found in neuroblasoma. The v-ras proteins were shown to have binding activity for GTP, and can convert GTP to its di or mono-phosphate forms. The enzymatic activity of the v-ras protein was less than that of the c-ras protein. During viral transduction, one nucleotide change has been observed with substitution of a different aminoacid from the c-ras protein. The changes in the ras protein at aminoacid residues 12, 13 or 59-61 have led almost invariably to a change in the oncogenic potential. GTPase activity is a regulatory factor, and the diminished GTPase activity of mutated ras proteins could lead to sustained effects on cell metabolism (Teich, 1986).

The sis oncogene was found in simian sarcoma virus and its protein product was in the cytoplasm. The amino acid sequence of the platelet derived growth factor (PDGF) was homologous to the protein product of v-sis. PDGF (c-sis) is in platelets, which are released in response to a wound, delivering PDGF to that area to stimulate endothelial or epithelial growth to heal the wound. There was also homology between the protein product of v-erbB and the amino acid sequence derived from Epidermal Growth Factor Receptor, c-erbB (Teich, 1986).
The *myc* oncogene, as isolated from four different avian sarcoma viruses, is a multi-gene family. The cellular *c-myc*, with its homologue *v-myc*, and two other different but related genes, *N-myc* and *L-myc*, are found in neuroblastomas, retinoblastomas and in small cell lung carcinomas (Teich, 1986).

Southern blotting, PCR, DNA sequencing, dot-blot hybridization, Western blotting and immunohistology have allowed these genes and their proteins to be analysed in clinical samples from patients with different types of tumours. Discovering more oncogenes and obtaining information on the expression of these genes could allow the prediction of the course of tumour progression in any individual, and this will also provide the target for pharmacological and immunological treatment. (Gullick and Sikora, 1990).

Higher levels of circulating oncoproteins can be used as tumour markers in patients with suspected malignancies. Antibodies to oncogene products have also been used for tumour localization. For example, antibodies to EGF receptors have been used to identify gliomas. The discovery of oncogenes provides new targets for drug development, including examples such as growth factors, growth factor agonists and antagonists, and antibodies to the receptors of growth factors. (Gullick and Sikora, 1990).
1.2.4. Tumour suppressor genes

Tumour suppressor genes are negative regulators of cellular proliferation, and their inactivation results in loss of a crucial brake on tumour growth (Vogelstein, 1993). In order to exert an effect, both alleles must be deleted or mutated, and cancer results when no normal copy is present (Knudson, 1986).

Some examples of tumour suppressor genes which are lost in human tumours are:

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Gene</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma</td>
<td>RB</td>
<td>chromosome 13q14</td>
</tr>
<tr>
<td>Wilm's tumour</td>
<td>WT-1</td>
<td>11p13</td>
</tr>
<tr>
<td>Neurofibromatosis</td>
<td>NF-1</td>
<td>17q11</td>
</tr>
<tr>
<td>Li-Fraumeni syndrome &amp; others</td>
<td>TP53</td>
<td>17p12-13</td>
</tr>
<tr>
<td>Familial adenomatous polyposis coli</td>
<td>APC</td>
<td>5q21</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>DCC</td>
<td>18q21</td>
</tr>
</tbody>
</table>

If one allele of a tumour suppressor gene is inactivated in the germ-line, this results in a predisposition to cancer, as the second allele can undergo somatic mutation at the site of the tumour. Alternatively, both alleles can undergo somatic mutation leading to sporadic tumours (Vogelstein, 1993).

The first evidence of the importance of tumour suppressor genes in human carcinogenesis came from a study of the familial childhood cancer, retinoblastoma, by Knudson in 1971. Retinoblastoma is one of many heritable childhood tumours. It is a malignant tumour of the developing retina and appears in early childhood. There are two forms of retinoblastoma, the bilateral form, which occurs in about 25-30% of cases and is diagnosed at an earlier age, around 11 months, and the unilateral form, which occurs in about 70-75% and is diagnosed later at around 2 years of age. The disease can be either familial or sporadic. The familial form is inherited as an autosomal dominant condition with high penetrance of 90%. In this group, 68% of cases are bilateral and 32% unilateral. On the other hand, the sporadic variety is seen when the disease affects only one member of a family, and it is nearly always unilateral. Sporadic unilateral forms are usually caused by two somatic mutational events.
occurring in retinal cells, and there is no germ line mutation. Hereditary retinoblastoma, which includes some sporadic bilateral cases, arises from an additional somatic mutational event in a retinal cell which carries the inherited mutation (Cavenee et al., 1983). There are two groups of genetically predisposed children, those who inherit the tumour potential with a dominant mode of transmission, and those who have a deletion of 13q in all of their somatic cells. The second group has a specific chromosome abnormality which occurs prezygotically, and consistently predisposes to a specific tumour (Knudson, 1976).

As shown in the diagram below, the hereditary form of the disease arises as a germ line mutation of the Rb locus (a) and is inherited by an individual who carries such a mutation in all of their somatic and germ line cells (b). Any subsequent event in a predisposed retinal cell, which results in homozygosity for the mutant allele at the Rb locus on both chromosomes 13, will result in development of a tumour. Possible mechanisms are: mitotic non-disjunction with loss of the wild type, resulting in hemizygosity at all loci on chromosome 13 (c); mitotic non-disjunction with reduplication of mutant chromosome, resulting in homozygosity at all loci on the chromosome (d); mitotic recombination between the Rb locus and the centromere, resulting in heterozygosity at loci in the proximal region; and homozygosity in the rest of the chromosome including the Rb locus (e), gene conversion (f), deletion (g) or mutation (h).

Cavenee et al., 1983
The Rb gene encodes a nuclear phosphoprotein of 105 kDa (Lee, 1987). The authors suggested that the protein may be involved in growth regulation in a wide variety of cell types. It is expressed in all human cells except certain types of tumour cells with Rb gene inactivation.

The Wilms' tumour gene, WT1, codes for two slightly different proteins, which are transcription factors. Allele deletions or mutations within the zinc finger regions of the gene cause childhood renal tumours. (Evans, 1993 (Review) ; Little et al., 1992).

The NF1 gene predisposes to inherited neurofibromas and sarcomas. This gene codes for a 250 kDa protein, which has a GTPase activating domain that down-regulates the ras oncogene. Homozygous inactivation of NF1 in patients with malignant neurofibromas is the evidence for NF1 being a tumour suppressor gene. (Evans and Legius et al., 1992).

In colon cancers, the inheritance of a mutated APC gene is not responsible for all forms of the disease. Individuals heterozygous for this mutation develop hundreds of colon adenomas in adolescence, a condition known as familial adenomatous polyposis. These individuals have a very high risk of developing colon cancer (Vogelstein, 1988). Loss of the APC gene due to somatic mutation is also important as an early event in the development of sporadic tumours. Two genes implicated in the progression of colon cancer, DCC (deleted in colorectal cancer) and MCC (mutated in colorectal cancer) have been isolated and shown to be altered in colon cancers. (Evans, 1993 (Review) ; Fearon et al., 1990).
1.2.4.1. Loss of Heterozygosity (LOH)

The loss of the normal tumour suppressor gene, in the presence of a mutated homologue, will reveal a defective gene and allows its unopposed dysfunction. A variety of mechanisms such as whole homologue loss, or mitotic recombination or deletion, could result in loss of the normal gene. These can be revealed by loss of heterozygosity (LOH) at one allele of a heterozygous locus (Osborne and Leech, 1994).

For known and putative tumour suppressor genes, analysis of all relevant loci is achieved by two means, by direct visualisation of chromosomes and by allele loss studies or allelotyping, involving every arm of every chromosome (Osborne et al., 1994).

In allelotyping by restriction fragment length polymorphism (RFLP) analysis (Sato et al., 1991; Cliby et al., 1993), labelled polymorphic probes are used as markers to identify individuals heterozygous for specific chromosome loci. Alleles present in tumour DNA from heterozygous individuals can then be compared with those present in the corresponding constitutional DNA, to detect allele loss or loss of heterozygosity (LOH). Such allele loss or LOH in the vicinity of tumour suppressor gene loci has now been observed in a high proportion of human solid tumours (Busby-Earle et al., 1993).

The RFLP method has many limitations, such as the low informativity of many loci, the limited availability of a number of RFLP markers, and the requirement for relatively large amounts of tumour DNA. The recent development of large numbers of microsatellite polymorphic (MSP) markers facilitate a more comprehensive allelotyping. They are highly informative, well distributed and need only very small samples. Allelotyping can also be performed rapidly by MSPs (Osborne et al., 1994). The following studies are presented here as examples.

In 1993, Foulkes et al. examined 29 cases of ovarian carcinoma using 19 probes by RFLP on chromosome 6. LOH on 6q was 55% and 6p was 28%. Of the former 55%, 63% showed loss of all informative markers on 6q. It was suggested that a larger series should next be examined.
The following year, in 1994, Osborne et al. examined 25 cases of epithelial ovarian tumours, using a set of microsatellite polymorphisms. More than one MSP was used per chromosome arm, and the mean informativity was 85.2%, ranging from 64-100%. A high frequency of allele loss (40%) was found on 5q, 48% on 9q, 43% on 11p, 46% on 14q, 40% on 15q, 61% on 17p, 64% on 17q, 45% on 19p and 40% on Xp. This study confirmed the previous findings at some sites, and also suggested new sites for tumour suppressor genes in ovarian cancer.

Samples from sixteen patients with hepatocellular cancer (HCC) were analysed by Southern blotting with 22 RFLP probes. Seven patients did not have cirrhosis. Seven patients with liver secondary metastases from colorectal primary tumours were included in this study, in order to compare the outcome with seven non-cirrhotic HCCs. LOH was seen on 5q35-qter in patients with non-cirrhotic HCC, and on 5p in patients with cirrhotic HCC. LOH in patients with liver secondaries from colorectal primary tumours was on 5q21-22. This study suggested that a tumour suppressor locus for non-cirrhotic HCC could be on chromosome 5 but a different locus from Familial Adenomatous polyposis coli (Ding et al., 1991).

Loss of heterozygosity studies in small cell lung cancer cases have shown a high incidence of allelic deletions at three different loci, 3p (100%), 13q (91%) and 17p (100%). LOH on 3p and 13q occurred prior to N myc amplification, and LOH on 3p was also detected in 83% of adenocarcinomas (Yokota et al., 1987).

Although previously no loss of alleles on chromosome 13 in adenocarcinoma and medullary carcinoma of the breast had been found, four out of ten cases of ductal breast cancer were found to show LOH on chromosome 13 in a study by Lundberg et al. in 1987. Of that four cases in this study, two showed specific loss on chromosome 13 at three loci. The third case had LOH on chromosomes 2, 14 and 20 along with chromosome 13. The fourth cases had LOH on chromosomes 5 and 13.
1.2.5. Specific chromosome rearrangements in cancer

The breakpoints of recurrent and consistent chromosome aberrations in a number of malignancies have now been identified as sites of new and already known proto-oncogenes. This has confirmed the pivotal role of chromosome rearrangements in tumour development (Rabbitts, 1994)(Review).

Two cytogenetic changes are particularly important in carcinogenesis; deletion and translocation. At the molecular level, deletion can result in the loss of a tumour suppressor gene. Specific translocations can lead to two principal changes. Either the gene for a T-cell receptor or an immunoglobulin protein comes into close proximity with a proto-oncogene and activates it, or breakage and fusion occur within two genes on different chromosomes leading to a fusion gene encoding a chimeric protein. The genes involved encode transcription factors, indicating that altered transcription plays a major role in tumourigenesis (Rabbitts, 1994)(Review).

Chromosome translocation leading to gene fusion, resulting in the synthesis of chimeric proteins, was first observed in 1960 by Nowell and Hungerford, who discovered the Philadelphia chromosome in chronic myeloid leukemia. In most cases, a small abnormal chromosome 22 results from a reciprocal translocation of the terminal part of chromosome 9q onto the breakpoint of chromosome 22q. The t(9;22) in CML involves a break at the 5' end of the abl oncogene on chromosome 9, and translocation of the region distal to this onto a break site at the break point cluster region (BCR) gene on 22. The normal product of the abl gene is a 145 kDa protein which has tyrosine kinase activity and is expressed in proliferating cells. The normal form of the abl gene is essential for normal cellular activity. The translocation in CML results in a mutated cellular oncogene which is transcribed into a 210kDa protein. Sequences within the first exon of the BCR gene are essential for the oncogene activity of the fusion protein (Evans, 1993)(Review). The BCR gene has kinase activity and can phosphorylate several different protein substrates (Maru et al., 1991). The translocation brings these two abl and bcr genes into juxtaposition, enhancing tyrosine
kinase activity, and driving the cells containing the translocation into continued proliferation (Heisterkamp et al., 1990).

The best example of proto-oncogene activation due to translocation is seen in Burkitt's lymphoma. It is associated with one of three different translocations involving chromosome 8 and one of the three immunoglobulin genes. Any one of these translocations places the myc oncogene on chromosome 8 in close juxtaposition to the immunoglobulin genes. Burkitt's lymphoma is a B cell malignancy. B cells produce immunoglobulin and Ig genes are very active. The myc gene is an early growth response gene. Therefore the myc gene is switched on in proliferating cells and switched off in quiescent cells. The activated myc gene will turn on a series of genes which are responsible for cell proliferation (Evans, 1993) (Review). The translocation, t(8;14)(q24;q32), moves the c-myc oncogene close to the immunoglobulin heavy chain (H) gene. This is found in 90% of cases. Two other variant forms of translocation in Burkitt's lymphoma are t(2;8) and t(8;22), which both juxtapose the c-myc to the immunoglobulin light-chain (L), k or \( \lambda \) genes respectively (Rabbitts, 1994).

Most of the follicular B-cell lymphomas involve translocation of the IgH on chromosome 14q32 to 18q21. The association of the bcl 2 gene at 18q21 with the J segment of IgH results in an overproduction of bcl 2 protein. The bcl 2 protein prevents cell death, and resulted in the continuous proliferation of the B cells (Vaux et al., 1988).
1.2.6. Specific chromosome rearrangements in solid tumours

The studies on chromosomal rearrangement in solid tumours are fewer than studies on haematological malignancies. This is mainly an account of the difficulties in obtaining sufficient numbers of dividing cells, and also owing to the greatly increased numbers of chromosomes in solid tumours. Recently, firstly because of the increased use of enzymes for dis-aggregating tumour tissue samples, secondly the addition of growth factors and feeder cells to tissue cultures, and thirdly following the new developments in molecular and cytogenetic techniques such as FISH and CGH, cytogenetic information on solid tumours have improved considerably (Sheer, 1986). The following studies on various solid tumours are discussed as examples.

The isochromosome for the short arm of chromosome 12, i(12p), is characteristic of testicular germ-cell tumours (De Jong et al., 1990). More than one copy of the i(12p) is associated with the more undifferentiated tumours with a worse prognosis. A number of testicular germ-cell tumours have other chromosome 12 rearrangements instead of, or in addition to, i(12p). Some of these have been shown by FISH to result in over-representation of 12p sequences even in the absence of an i(12p) (Suijkerbuik et al., 1993; Al Jehani et al., 1995). The chromosome region rearranged with 12p could be important for alteration of oncogenes and / or tumour suppressor genes in i(12p) negative cases (Parrington et al., 1994).

Liposarcoma is one of the most common soft tissue sarcomas in adults. With the latest molecular genetic technique, Comparative Genomic Hybridization (CGH), a gain of 12q has been found to be consistent in well differentiated liposarcomas (Szymanska et al., 1996). The most common copy number increase was at 12q13-14, and 43% of tumours had high level amplification of 12q15. Several oncogenes in human sarcomas have been mapped to 12q13-14 (Szymanska et al., 1996).

Small round cell tumours in children and adolescents such as neuroblastoma, Ewing's sarcoma, peripheral neuroectodermal tumour and rhabdomyosarcoma are frequently
difficult to diagnose, especially in the undifferentiated form. Since their histology can be identical, finding a specific chromosomal abnormality in such tumours is invaluable for correct diagnosis and treatment. The t(11;22)(q24;q12) is present in the majority of cases of Ewing's sarcoma, and in many peripheral neuroectodermal tumours. This is very important diagnostically (Taylor et al., 1993).

Lung cancer is a common malignancy in adults, and so far two types of genetic change have been reported in cases of small cell lung cancer. One is the amplification of myc genes, and the other is deletion of part of the short arm of chromosome 3 (Yokota et al., 1987).
1.2.7. Chromosome abnormalities in Cervical cancer

Cytogenetic studies on cervical cancer using chromosome banding techniques were still deficient even a decade after the advent of such banding. This was probably on account of the technical difficulties in producing a large number of good quality metaphases, and in establishing cervical cancer cell lines (Atkin et al., 1982). In 1982, Atkin et al. using G and C banding on direct preparations from nine cases of primary carcinoma of the cervix, showed structural changes involving chromosome 1 in most samples (6 out of 9), chromosome 11 in 5 out of 9, chromosomes 3 and 6 in 3 out of 9, and chromosome 17(17p+) in 2 out of 9. A small metacentric chromosome, thought to be 5q+, was seen in 5 out of 9 samples. Trisomy without any structural changes was seen in chromosome 1 in one case and trisomy 3 in 4 cases. Chromosome losses occurred in 13, 21, 18 and X.

Atkin and co-workers presented more data in 1984 on non-random chromosome changes in direct preparations on 10 cases of cervical tumour. Yet again, chromosome 1 was the chromosome most commonly involved in structural rearrangements (7 out of 10 cases) followed by chromosome 11 (5 out of 10 cases). A small metacentric chromosome, thought to be either chromosome 4 or 5, a long arm deletion or a short arm isochromosome often seen in duplicate, was observed in 6 out of 10 tumours. Chromosome 17 abnormalities were seen in 4 tumours and chromosome 2 abnormalities in 2 cases.

Five years later, in 1990, Atkin et al. performed another big study on 43 cases of cervical cancer. This time the most common abnormality in 77% of the samples was an i(5p) or an i(4p) often in duplicate. Chromosome 1 structural rearrangements were found in 60% of cases (ie either an i(1q) or 1p- or translocation of part of 1q onto another chromosome), chromosome 17 in 47% of cases, chromosome 11 in 37% of cases, chromosome 3 in 26% of cases and chromosomes 2, 6, & 9 in 19% of cases.

In 1991, Sreekantaiah et al. conducted a cytogenetic analysis on 150 cervical cancer samples using banding techniques. Chromosome 1 abnormalities again represented the most common karyotypic change in this study. Chromosome 1 abnormalities have been reported in a wide
variety of malignancies, including leukemia, lymphoma, melanoma, breast cancer, lung cancer and neuroblastoma, suggesting that chromosome 1 plays a particular role in tumour progression.

In 1992, Wang et al. analysed 12 cases of cervical cancer by direct preparation, using G banding. Chromosome 1 was the most frequently involved, and chromosome 1, 2, 3, 4, 5, 10, 15, 16, 17, 19 and 20 were over-represented, which again supports the view that chromosome 1 is especially involved in tumour progression. However, Popescu and Dipaolo pointed out in 1992 that chromosome 1 aberrations in cervical cancer could be an early event, contributing to cell immortality. Additional deletions of 11p, 17p and 3p, involving tumour suppressor genes may be required to complete the tumour development.

In 1994, Mitra et al. observed i(5p) and 6q as common and non-random changes in two newly established cervical squamous cell carcinoma cell lines. Because i(5p) had been reported previously (Atkin) and because it occurred in both of the cell lines in this study, the authors have suggested that this i(5p) may be the specific lesion for cervical cancer, analogous to i(12p) in testicular germ cell tumours.

In 1994, Atkin et al. reported finding identical chromosome 17 rearrangements in two squamous carcinoma cell lines, in which the whole 17p had been replaced by the long arm of chromosome 22 (17;22)(q10;q10). In their previous work published in 1989 as mentioned above, they had described 17p+ in more than 40% of their cases, although they had not been able to identify the translocated material on the chromosome short arm at that time. The authors believed that chromosome 17 rearrangements could be significant steps in cervical cancer development.

Studies carried out over the years on the HeLa cell line D98/AH-2 as reported by Miller et al. (1971), Nelson-Rees et al. (1974 and 1980), Francke et al. (1973), Bengtsson et al. (1975), Heneen, 1976 and by Stanbridge et al. (1981), using conventional cytogenetic staining techniques, could not clarify the origin of most of the marker chromosomes (Ruess et al., 1993). More than half of the HeLa cells have 61 chromosomes, the range being
between 58 - 63. Most of the structural rearrangements involved chromosomes 1, 7 or 8. Numerical aberrations involved chromosomes 15 and 16. There were 15 different chromosome markers, and in Ruess's study, using FISH, the origin of most of the markers (13 out of 15) could be identified with certainty by chromosome painting. Some authors observed that some abnormal chromosomes revealed by G-banding were not confirmed by painting. Likewise, some abnormal chromosomes detected by painting were not found in G-banded karyotypes. The discrepancies can be explained partly by the inadequate resolution of the G-banding and partly by the quality of the DNA probes.

Ruess et al. observed that small fragments (less than 10Mb) were unlikely to be detected unless cosmids or Yeast Artificial Chromosomes (YAC) were used. The marker, M5, der(14)t(12;14), appears to be unique for D98/AH-2. Previously marker M8 had been identified as a normal chromosome (eg. a normal X by Francke et al., 1973). Marker M13 had been interpreted as a t(8;22), but the translocated part of chromosome 8 had been too small to determine its region of origin, on the basis of its banding pattern alone. In M15, only chromosome 19 material was found, but this appeared to be smaller than a normal chromosome 19, and the centromeric heterochromatic region was reduced in size. The banding pattern of this marker did not allow the breakpoint of the deletion to be defined. Marker M3, der(2)t(2;9) has never been identified as a marker in any previous publications (Ruess et al., 1993).

In 1995, Jesudasan et al. investigated with FISH eight cervical cancer cell lines, HeLa, SiHa, Caski, C4-I, Me180 & M751 (all HPV +ve) and two HPV -ve lines, C33A & HT3. The INT2 YAC, and the INT2 cosmid derived from this YAC on chromosome 11, were used in this investigation. A breakpoint within the sequences spanned by the INT2 YAC was found in the HeLa and Caski cell lines. The INT2 cosmid was deleted in SiHa and C33A cell lines. However, the SiHa and C33A lines retained cosmid sequences of Cyclin D1, localised 100kb proximal to INT2. The authors have suggested that this deletion was significant, and that the 100kb interval between the two cosmids, Cyclin D1 and INT2, might possibly contain the potential tumour suppressor gene for cervical cancer. In the same
year, Uejima et al., 1995 working on the cell line SiHa, inferred that the normal human chromosome 2 could carry a gene or genes that could induce cellular senescence in cervical cancer.

In 1996, Heselmeyer and co-workers (collaborative study of NIH, John Hopkins University, Sweden and Germany) published a very interesting study on the evolution of chromosomal abnormalities in cervical cancer. In this investigation the Comparative Genomic Hybridization technique was applied to normal cervical epithelium, and to cervical samples with mild dysplasia, moderate dysplasia, severe dysplasia and invasive cervical tumour. Apart from tetraploidy, there were no recurrent chromosomal aberrations observed in the normal epithelium, or in the mild and moderate dysplasia samples. This indicates that tetraploidy precedes the specific chromosomes gains and losses. 10% of the severe dysplasia samples showed a 3q gain. However, 3q was over-represented in 90% of the invasive tumour samples. Therefore, the authors have concluded that changes in 3q could be the pivotal genetic aberration at the transition from severe dysplasia to invasive cervical carcinoma.
1.2.8. LOH in cervical cancer

1.2.8.1. Tumour suppressor genes in HeLa

Somatic cell hybrids, fusion between two or more different cells of the same or different species, are very helpful in genetic investigations of tumours (Misra et al., 1989). By observing the human cell hybrids resulting from fusion between tumourigenic HeLa cells and normal cells, Stanbridge showed in 1976 that tumourigenicity behaves as a recessive trait. In 1981 Stanbridge et al. observed once again the correlation between the loss of a single copy of chromosomes 11 and 14 and the reappearance of tumourigenicity in these hybrids. In 1980 and 1982, Klinger confirmed these findings, by using HeLa and fibroblast hybrids. Both Srivatsan et al. and Kaelbling et al. agreed in 1986 that the loss of a single copy of normal chromosome 11 is sufficient for the re-expression of the tumour.

The conclusive evidence came from Saxon et al. in 1986, that a normal chromosome 11 was involved in tumour suppression, and they were able to show tumour suppression by the introduction of a normal chromosome 11 into a tumourigenic HeLa/fibroblast hybrid cell by the microcell transfer technique. These studies therefore indicate the presence of tumour-suppressor sequences which are important in cervical cancer, on normal chromosome 11.

Extensive RFLP analysis of various non-tumourigenic and tumourigenic hybrids, with at least 50 different chromosome 11 specific probes, has been used to determine the location of tumour suppressor gene(s). This has shown that the gene involved in the suppression of the HeLa cell tumourigenicity is localized on the long arm of chromosome 11, region q13-23 (Misra et al., 1989).
1.2.8.2. LOH in primary cervical tumours

In 1991, Srivatsan et al. studied 33 primary cervical tumour samples with RFLP, using chromosome 11 specific polymorphic DNA markers. Comparative analysis of tumour DNA with normal DNA was performed in 15 cases. Six of these 15 had deletion of chromosome 11 sequences. Four of the six were informative for both 11p and 11q. Two cases showed LOH at 11p15-q13, with the possibility of the loss of a complete copy of chromosome 11. Two samples showed preferential loss of 11q with retention of heterozygosity for 11p15-p13 and 11p14 - cen. Two more samples showed LOH at 11q13 and LOH 11cen-q23. Eighteen tumour samples, for which normal DNA was not available for comparative analysis, were also included in this study. Two samples contained homozygous alleles for 11 different probes at p15-q23, and at q23. The rate of overall LOH for chromosome 11 in cervical cancer in this study was 30% (Table 1). The authors suggested that their results agreed with those of Saxon et al., 1986, which indicated a role for chromosome 11 in tumour suppression of HeLa cervical carcinoma cell lines. Of the five primary cervical tumours with chromosome 11 deletions, one showed LOH on 3p, and one on 14q. Three cases with heterozygosity for chromosome 11 were also heterozygous for 3p and 14q. The authors suggested that the chromosome 11 deletions could have preceded the 3p and 14q deletions (Srivatsan et al., 1991).

In 1992, Kaelbling et al. analysed 27 cervical cancer samples as well as normal DNA from the same patients. Southern blotting was applied using polymorphic probes at 17p13, including the TP53 locus. Four of the 27 had LOH at 17p13 (15%)(Table 1). The authors referred to previous studies by Atkin et al, regarding structural changes on 17p in cervical cancer (mentioned in 1.2.7). They have suggested that in HPV -ve cervical cancer, the absence of wild type TP53 could be due to the loss of the 17p13 or TP53 locus, leaving the cell with only mutant TP53. They failed to find LOH (mutation of TP53 ) in 4 HPV -ve cases in this study, but this did not change the authors' view, and they expressed a particular interest in determining further the precise status of TP53 in these 4 HPV -ve cases without LOH (1.2.12.). These authors also observed LOH on 11p and 11q in their study.
In 1992, Jones and Nakamura, examined eight cervical cancer samples, 13 endometrial cancer samples, and 4 ovarian cancer samples, a total of 25 cases. The markers were informative in 65% of the cases examined. LOH was seen in 52% of overall cancer samples in the study. However, LOH was seen in 75% of the cervical cancer samples (Table 1), in 38% of the endometrial cancer samples and in 50% of the ovarian cancer samples. The authors' observations of loss or retention of heterozygous loci indicated that a common region for LOH in cervical cancer was at 3p13-14.3. LOH in endometrial cancer was at 3p13-21.3. Accordingly, the authors' conclusion was that there is some overlap of the regions for endometrial and cervical cancer, whereas a separate LOH region for ovarian cancer was at 3p21.1 - 22 (1.2.4.1.). The authors suggested that region 3p13-14.3 could either have a single tumour suppressor gene or a cluster of tumour suppressor genes, which are involved in different types of gynaecological tumours.

In 1993, Kohno et al. set up deletion mapping of 3p on 47 cervical cancer samples. 35 were squamous carcinomas, 10 were adenocarcinomas and 2 were adeno-squamous carcinomas. 12 cases were at stage I, 27 were at stage II, 6 were at stage III and 2 were at stage IV. LOH on 3p was detected overall in 21 out of the 47 specimens (45%) (Table 1). In this study, a common region for LOH was 3p13 - 3p21, and the highest frequency for LOH was at 3p21 among 24 markers analysed. The conclusion reached was that there may be a novel tumour suppressor gene at 3p13-21. The authors observed that the incidence of LOH was similar in squamous cell carcinomas (46%) and in adenocarcinomas (50%), and there was no correlation between pathological staging and the prevalence of LOH on 3p.

In 1993, Busby-Earle and co-workers presented data on LOH in twenty cervical cancers, using 22 RFLP markers. In their study, of 211 cases analysed with informative loci, only 22 showed LOH (10%), in nine tumours. However, 11 of 20 tumours did not show LOH. Six markers on chromosome 17 at p13 (including TP53 locus) assisted detection of LOH in 3 of 20 cases (15%) (Table 1). The findings of this study supported those of Kaelbling et al., 1992 (above) and the authors suggested that an association between 17p LOH and cervical cancer was not as strong as in the cases of other human tumours. No LOH was found on
17q in 13 informative cases, and none on 13q at the Rb gene locus in 14 informative cases. LOH was found in 5 of 19 cases (26%) (Table 1) on 11p, in three cases at 11p15 and the remainder at 11p15-ter. No loss with markers at 11p13 was observed in four informative cases. The authors referred to Srivatsan et al., 1991 (above), where LOH was found in 30% of cases on chromosome 11p. Moreover, they noted that Riou et al., 1988 had reported finding LOH, in 36% of his cases on 11p (not in the Table 1). They considered it unlikely that there were important tumour suppressor gene(s) for cervical cancer on chromosome 11p. Chromosome 11q was not examined in this study. Six markers on chromosome 5 at q21 (region of APC & MCC) showed LOH in 5 of 16 cases (31%). LOH on chromosome 18q (DCC gene locus) was seen in 3 of 12 cases (25%) (Table 1). LOH on chromosome 3 at p21 had been seen in one of four cases of cervical tumour (25%). This contrasts with a study by Yokota et al., 1989, which reported the finding of LOH at 100% on chromosome 3 at p14-21. However, Busby-Earle did not use the same probes as Yokota et al., and LOH was found in 14% on chromosome 8q (in one of seven cases).

In 1994 Mitra et al. reported a study in which they had found LOH on 53 primary cervical cancer samples by Southern blotting, using 57 polymorphic probes. They had examined probes on 11 chromosome arms, including 5p and Xq. They reported that the overall prevalence of LOH in the cases they studied was >25%. Specific LOH prevalence rates were: on 1q 26%, on 3p 35%, on 3q 31%, on 4q 46%, on 5p 33%, on 5q 38%, on 6p 28%, on 10q 28%, on 11p 42%, on 18p 38% and on Xq 26% (Table 1). LOH was observed quite frequently on 4q and 5p and the authors suggested that in their study there was strong evidence for candidate tumour suppressor genes at 4q21-23, at 5p proximal to 5p15, and between Xq22 & Xq11-13 (Table 1).

Karlsen et al., 1994 examined 31 paraffin-embedded cervical cancer samples for prevalence of LOH at 3p21. Of 31, 20 were HPV +ve. 14 of these 20 HPV +ve cases showed LOH (70%). Five of HPV -ve cases showed LOH (45%). More of the HPV +ve tumours had LOH as compared to the HPV -ve tumours showing a weak association of LOH and HPV integration. Of 31 cases, 27 were informative and 19 showed LOH (70%). LOH at 3p21
was found in 40% of cases and 3p21.2-21.3 was found in 44% (Table 1). These results were compared with those of Kohno et al in 1993 (above), in which 45% showed LOH at 3p21.

In 1994 Jones et al. analysed 35 tumour samples as well as normal tissues from each of the same patients. Of the 35, 21 were cases of uterine endometrial carcinoma. No loss was found on 4p, 8q, 18q, 19p or 20p. LOH on 7q was found in 23%, 9q in 38%, 10q in 33% and 17p at TP53 in 36%. The authors observed that there were no significant differences between cervical and endometrial cancer with respect to LOH at a given locus (Jones and Nakamura, 1992) (above). Of results referring to cases of combined cervical and endometrial cancer, LOH was found in 71% of them at 3p21 and in 61% of cases at 3p13. Among cervical cancer samples, LOH prevalence on chromosome 3p at 3p21 ranged from 100% (D3S32) to 50% (D3S685), and at 3p13 was 86%.

In the same year (1994) Hampton and co-workers studied LOH on chromosome 11 in 32 primary cervical tumour samples, using 16 polymorphic markers. Five markers on 11p showed LOH in 28% by visual assessment, but only in 16% when densitometric analysis was used. In the 5 cases with LOH on 11p, this was accompanied by LOH on 11q. The authors suggested that these two events, LOH on 11p and 11q, were independent, and that LOH on 11p occurred subsequently to LOH at 11q. LOH on 11q was found in 62% (20 of 32) by visual assessment. 14 of 20 cases (44%) showed LOH by densitometry. The highest frequency of LOH was at 11q23, which was found in 43% to 52% dependent upon polymorphic loci. The LOH frequency was low at 11q12 (LOH was found in only 10% of the cases). It was therefore suggested that any relevant tumour suppressor gene(s) for cervical cancer could be at 11q22-24. In this study, LOH on 2p was found in 19%, 20q in 8% and 8q in 14% and no loss was found on 17q.

Recently, in 1996, Mullokandov et al. examined 38 cervical cancer samples by PCR using 75 polymorphic microsatellite loci on the arms of most chromosomes. LOH was found on 19 chromosome arms and the overall prevalence was 20-43%. Frequent LOH was found on chromosome 3 at p13-25.3 (in 39% of cases), chromosome 6 at p21.1-23 (43%) and
chromosome 18 at q12.2-21.2 (35%). LOH on 11p was found in 16% and 11q was found in 22%. LOH including TP53 locus was present in 15%. Chromosome arms, 1q, 2q, 3q, 4p, 4q, 5p, 5q, 6q, 7q, 8p, 8q, 13q, 16p, 18p, and 19p showed a LOH of 20-33%. The authors compared their results on chromosome 3 with those of Mitra et al. (1994), Jones et al. (1992 and 1994), Kohno et al. (1993), and Karlsen et al. (1994) (above). They suggested that putative tumour suppressor gene(s) could map to 3p22.1-24.1 and 3p losses could be a critical transformation and/or this could also be for progression, invasion and metastasis. Subsequently, Mullokandov compared his own results with those of Hampton et al. (1994) and Mitra et al. (1994), and suggested that possibly many different genes with tumour suppressor functions on chromosome 11 played a role in cervical cancer. Diverse LOH patterns in cervical cancer have indicated that different cervical carcinomas probably arise and progress on account of the loss of different tumour suppressor gene(s). The summary of all the above studies is shown in Table 1.

Recently in 1996, Hampton et al. reported detection of loss of heterozygosity by comparison of normal and tumour using PCR-based microsatellite loci and this technique showed advantages over southern blotting-based techniques. In their study a series of loci on chromosome 4 were investigated on 58 cervical carcinoma samples and two regions of chromosome 4 were commonly altered in cervical tumours (Chapter 4) (The data is not in Table 1).
| First author | year | sample size | 1p | 1q | 2q | 3p | 3q | 4q | 5p | 5q | 6p | 6q | 7q | 8q | 9q | 10q | 11p | 11q | 13q | 17p | 17q | 18p | 18q | 19q | 20q | Xq |
|--------------|------|-------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|---|
| Srivatsan    | 1991 | 33          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Kaelbling    | 1992 | 27          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Jones        | 1992 | 25          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Kohno        | 1993 | 47          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Bushby-Earle | 1993 | 20          | 25 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             | 25 | 31 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Mitra        | 1994 | 53          | 26 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             |    | 40 | 31 | 57 | 33 | 38 | 28 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Karlsen      | 1994 | 31          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Jones        | 1994 | 35          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Hampton      | 1994 | 32          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
| Mullokandov  | 1996 | 38          |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             |    | 14 | 25 |    | 29 | 39 | 22 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             |    | 39 | 22 | 29 | 20 | 21 | 43 | 22 | 33 |    | 26 |    | 15 |    |    |    |    |    |    |    |    |    |    |   |
|              |      |             |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |   |

Table 1: LOH in primary cervical cancer

(%) Frequency of LOH on various chromosome arms, comparative data from 1991-1996
1.2.9. Oncogenes in cervical cancer

**ERBB2 Oncogene**

Oncogene amplification has been investigated in 50 primary untreated cervical squamous carcinomas (Mitra et al., 1994). These samples originated from patients at clinical stages I and II, with histologically moderate to well differentiated carcinomas. Twenty-two proto-oncogene probes were used to analyse these samples. Amplification was observed for the ERBB2 gene in 7 cases (14%) and this ranged from 5 - 68 copies. Two tumour samples with ERBB2 amplification showed possible mutation of the gene. The authors suggested that ERBB2 could play an important role in cervical cancer tumourigenesis.

The ERBB2 gene encodes a transmembrane protein homologous to the epidermal growth factor receptor gene mapped to 17q21 (Mitra et al., 1994). In 1991, Pinion et al. showed that ERBB2 was over-expressed in over 60% of cases of CIN III and in invasive cervical cancer. A correlation between the degree of ERBB2 amplification and histological progression was not conclusive. Mitra et al. suggested in 1994 that it would be important in future to study the ERBB2 gene amplification and expression in various grades of CIN, in order to understand its role in cervical cancer evolution. They pointed out that ERBB2 amplification in CIN has not been reported before, and that Pinion and co-workers' observations had been quite encouraging for future study of CIN cases. This oncogene has also been reported to be amplified in poorly differentiated breast carcinomas (Adnane et al., 1989).

**PRAD1 gene**

In 1989 Arnold et al. investigated the PRAD 1 gene at 11q13 as a proto-oncogene in parathyroid adenomas, and suggested that it was linked to the BCL1 locus. The 11q13 had been regarded as a region of gene amplification in various solid tumours such as oesophageal cancers (Tsuda et al., 1989), head and neck squamous carcinomas (Schuuring et al., 1992 Berenson et al., 1989), breast (Schuuring et al., 1992) and bladder cancers (Proctor et al., 1991). PRAD 1, HST1, INT2 and EMS1 were identified in the same amplification unit, and the PRAD1 and the EMS1 were shown to be over-expressed in cells
with 11q13 amplification. (Komatsu et al., 1994). PRAD1 is also known as the Cyclin D1 gene.

In 1995 Kurzrock et al. reported a study of 10 cervical squamous carcinoma cell lines and 3 vulvar squamous carcinoma cell lines, using Southern and Northern blots to investigate the PRAD1 DNA and PRAD1 mRNA expression. All three vulvar cell lines showed PRAD1 mRNA over-expression, which was 10 fold greater than controls, and in addition two out of the three had a 5-10 fold amplification. PRAD1 abnormalities were seen in 4 out of 10 squamous cervical cell lines, with over-expression of PRAD1 in three and a rearrangement of PRAD1 DNA in one. Because of the amplification and rearrangement in PRAD1 DNA and over-expression of PRAD mRNA, it was suggested that deregulation of PRAD1, as a cell cycle regulatory gene, may be a significant molecular event in gynaecological squamous cancer evolution.

The myc Oncogene

The myc oncogene was first identified in avian myelocytomatosis viruses, retroviruses which cause acute myelocytic leukemia in chickens (Evan, 1990). The viral myc (v-myc) gene is closely related to the cellular myc gene from a normal cell, c-myc. This c-myc gene on 8q24 is highly conserved throughout evolution. The gene has three exons and the first is non-coding. Amplification of myc has been found in colon cancer, small cell lung cancer, retinoblastoma, neuroblastoma and breast cancer among others. Cells with an amplified myc gene frequently contain double minutes (DM) or homogeneously stained regions (HSR) (1.2.7.). Increased expression of myc is frequently related to the prognosis of the tumour. An over-expression of the c-myc gene in the early stages of invasive cervical cancers has been found to be associated with a higher risk of early recurrence (Couturier et al., 1991).

N-myc and L-myc share several regions of homology with the c-myc gene and encode similar sized proteins. During embryogenesis, N-myc expression is seen at a high level in fetal brain, kidney, retina and lung. Amplification of N-myc is seen in neuroblastoma, in Wilm's tumour, in retinoblastoma and in small cell lung cancer. In neuroblastoma, the N-myc protein is expressed at a high level, but this is not caused by amplification of the gene.
The C-myc and N-myc protein elevation in the cell is also due to other mechanisms, and amplification is only one of them. Therefore, the amount of N-myc protein is a better prognostic indicator than the degree of gene amplification (Evan, 1990).

The ras oncogene

The three ras genes in mammals are Harvey-, Kirsten- and N-ras, and they are highly conserved in eukaryotes, including yeast. The first 80 aminoacids are identical in Ha-, Ki- and N-ras. The second domain of 80 aminoacids are less conserved, and the third region is hypervariable. The ras genes encode 21 kDa proteins which are synthesized in the cytoplasm and attached to the inner surface of the plasma membrane. (Lemoine, 1990). In the past, several studies have shown that ras activation occurs in 40% of colorectal cancers. Large villous adenomas in the colon, with malignant potential, also contain activated ras oncogenes in 40% of cases, but less than 20% of small adenomas demonstrate such a change. In adenocarcinomas of the lung, ras mutations are seen in 30% of cases.

Although ras gene mutation was not found in 30 cervical tumour samples by Bos et al., 1988, Riou et al., 1984, found H-ras gene amplification in 8 out of 12 cervical tumour samples. It was suggested that there may be co-operation between the amplified T ras genes and Human Papilloma virus oncogenes. In 1988, Riou et al. investigated the c-Ha-ras-1 locus in cervical cancer, to determine its role in cervical tumour progression and its association with genetic factors. Identical c-Ha-ras-1 variable tandem repetition (VTR) alleles were detected in DNA from tumours and in matched control DNA samples. Heterozygosity for this allele was found in 28 of the 42 pairs tested, and the loss of one allele was found in 10 tumour samples from these 28 heterozygous patients. However, the frequency of LOH did not correlate with the stages of cervical cancer. In their study, 7 of the 10 cervical cancer samples with the c-Ha-ras-1 allele loss showed an activated c-myc gene. Similarly, all cervical tumour samples with c-Ha-ras-1 gene mutation showed an amplification or over-expression of c-myc gene. Activated c-myc gene involvement in cervical cancer progression had already been shown by Riou et al., 1984 and 1985. Likewise, the c-myc gene, being a strong prognostic indicator for the recurrence of cervical tumours, was observed by
Couturier et al., 1991 (above). Accordingly, Riou et al. suggested that the c-myc and the c-Ha-ras-1 genes cooperate in the progression of cervical cancer. They also indicated that it would be desirable to determine mutations in other members of the ras gene family in the future, and to study them in pre-invasive lesions.

1.2.10. Amplification

One form of chromosomal abnormality in tumours is gene amplification. This amplification may be found as a homogeneously staining region (HSR), or as double minutes (DM) (Teich, 1986).

Double minutes (DMs)

These are small, self-replicating, paired bodies containing chromosomal material. They have no centromere, so they segregate randomly at mitosis (Rabbitts et al., 1989).

Homogeneously staining regions (HSRs)

These occur within chromosomes, and they are recognised when the normal banding pattern of a chromosome seen after staining is interrupted by a region of homogenous staining. These regions are often very large, resulting in a chromosome which is noticeably larger than its homologue (Rabbitts et al., 1989).

HSRs and DMs are common features of solid tumours, but they are rare in haematological malignancies. (Sheer, 1986). DMs and HSRs are considered together because they are thought to represent the same gene. They appear to be cytological manifestations of a specific gene amplification. Different clones in cell lines may contain either DMs or HSRs, and the same gene has been localised to both structures (Rabbitts et al., 1989). Since these two forms are not seen in the same cell, HSRs and DMs are believed to represent alternate forms of the same gene amplification (Sheer, 1986; Nowell, 1992).

Amplification of myc genes has been reported in a number of tumours, including colon cancer, small cell lung carcinoma, retinoblastoma, neuroblastoma and breast cancer (1.2.9.).
Cells with amplified myc genes frequently contain DMs or HSRs. The degree of myc gene amplification varies from tumour to tumour, with a wide range of copy numbers. Most of the time myc gene amplification is seen in tumour cell lines and not in the primary tumours. However, in neuroblastomas HSRs or DMs have been seen in both cell lines and primary tumours. (Evan, 1990). Although N-myc maps to chromosome 2, amplified N-myc sequences have been observed in three different neuroblastoma cell lines as HSRs on chromosomes 4, 9 & 13. The chromosomal site of amplification is variable, and it has been suggested that DMs carrying amplified sequences integrate into different sites in the genome to form HSRs (Sheer, 1986). HSRs and DMs can therefore be found in chromosomes other than those in which the amplified genes are located (Rabbitts et al., 1989).

In vitro, DMs and HSRs can be artificially induced in cell lines which have become drug resistant, indicating that DMs and HSRs are the sites of the amplified genes responsible for the drug resistance (Rabbitts et al., 1989).
1.2.11. Human Papilloma virus infection in cervical carcinogenesis

In recent years, pre-invasive and invasive cervical carcinoma samples have been tested for HPV sequences by molecular biological and histopathological techniques. In 1995, Chang et al., reported that the PCR technique was more sensitive than Southern blotting for detecting HPV DNA in cervical cancer. In their study, by Southern blotting, HPV 16 DNA was found in 52.5% of the stage I and 63.3% of stage II cervical cancers. However, by PCR, HPV16 DNA in the same tumour samples was found in 87.5% and 93.3% of cases respectively. Concordance (both positive or both negative) between Southern blotting and PCR was 61.4%, and discrepancy (positive by PCR but negative by Southern blot) was 35.7%.

The viral genome contains coding regions called open reading frames (ORF). ORF are DNA sequences that encode for both structural and non-structural (regulatory) proteins. Structural proteins are coded by late genes (L), and non-structural proteins by early genes (E) in ORF. HPV types seen in human cells have seven E genes and two L genes. The E6 & E7 are involved in cervical carcinogenesis. The E6/E7 genes are involved in the regulation of viral growth, and have been shown in DNA transfection experiments to transform cell lines (Nair and Pillai, 1992)( Review).

In 1996 Hemstrom-Nilsson et al. suggested that HPV16 transcription in cervical cancer might be initiated in the E6 ORF and encoded by the E7 oncoprotein. This observation did not support the findings of Bohm et al. (1993) which suggested that the HPV 16 transcript was initiated within the E7 ORF.

HPV infection of the cervix originates in the basal cells, which are capable of mitosis. Once infection sets in, the virus remains either as a latent infection, or it undergoes active replication. The viral genome replicates extra-chromosomal as an episome, but, under certain circumstances, it will integrate with the cellular DNA (Nair and Pillai, 1992).
In 1991 Couturier et al. reported the investigation of the chromosomal localisation of the HPV 16 & 18 integration sites in 4 cervical cancer cell lines, IC1, IC2, IC3 & IC4. He observed that HPV integration sites were localised to chromosome bands which contained myc genes: c-myc (8q24.1) in IC1 (HPV 18), IC2 (HPV 16), IC3 (HPV 16), and N-myc (2p24) in IC4 (HPV 18).

In a later study by Gallego et al., 1994, it was shown that the HPV 18 integration site in the C4-I cervical cell line is at 8q22.1 on a derivative chromosome originating from an 8q;12q translocation.

In addition to the above, HPV integration sites have been determined in some other cervical carcinoma cell lines, including HPV 18 at 8q24 in HeLa, and at 12q13 in SW756. HPV integration sites in HeLa and C4-I are proximal to the c-myc gene at 8q24. The HPV 16 integration site in the SiHa cell line was at 13q21 (Durst et al., 1987).

The chromosomal region 8q24 appears to be a preferential integration site for HPV in cervical cancer. However, in studies so far, structural changes of 8q do not appear to be frequent in cervical cancer, and indeed have not shown a higher number of breakpoints than expected (De Braekeleer et al., 1992; Couturier et al., 1991). It is thought, therefore, that HPV integration above is an insufficient pre-condition for cervical cancer development.

Observations by a number of groups have suggested that HPV infection of cervical epithelium is an important event in the development of cervical dysplasia (CIN), but progression to invasive cancer appears to be dependent on further genetic changes. (Zheng et al., 1994; Mullokandov et al. and Larson et al., 1996)

In 1995 Nurnberg et al. suggested that c-myb may play a role in HPV-associated cervical carcinogenesis, since c-myb expression was increased in HPV-associated cell transformation.
1.2.12. TP 53 and the retinoblastoma gene in HPV positive and negative cervical cancer

The TP53 gene is on 17p13 and is composed of eleven exons. The product of TP53 is a 393 aminoacid nuclear phosphoprotein which is approximately 53 kDa in molecular weight. The TP53 is found in low amounts in normal cells because of its short half-life of about 6 - 20 minutes, and the wild type protein does not normally accumulate in amounts detectable by conventional methods (Chang et al., 1993)(Review).

In 1991 Scheffner et al. reported a study of five HPV +ve cervical carcinoma cell lines and two HPV -ve cervical cancer cell lines, C-33A and HT-3. All HPV +ve lines expressed normal Rb protein and low level wild type TP53 protein. Sequence analysis of TP53 cDNA did not show any mutation. But Rb and TP53 genes mutations were found in both HPV -ve lines. Elevated levels of TP53 protein were found in HPV -ve lines, because of the accumulation of inactive TP53, which resulted from mutation in the gene encoding TP53. E7 of the HPV forms complexes with the product of the Rb tumour suppressor gene (Rb). The E7 proteins of HPV 16 & 18 were observed to bind Rb ten times more than HPV proteins from low risk HPV types. The E6 protein of high risk HPV types, was seen to form complexes with the TP53 protein. The authors therefore suggested that their results support the hypothesis of normal function of Rb and TP53, either by mutation or by specific interaction of these proteins with E6 and E7.

In 1991 Werde et al. examined the expression of Rb and TP53 in eight cervical carcinoma cell lines, six of which were of HPV +ve and two of which were HPV -ve. Protein analysis showed abnormal Rb and TP53 expression in both HPV -ve lines, but no mutation was detected in HPV +ve lines. On sequencing, point mutations within the TP53 sequences were found in HPV -ve cases, but only wild type TP53 was found in HPV +ve lines.

In 1994 Busby-Earle et al. reported studying 47 primary cervical carcinomas (36 were HPV +ve) for the mutational status of the four hotspot regions of p53 gene, by PCR and denaturation gradient gel electrophoresis (DGGE). The HPV status was also determined by
PCR. LOH on 17p13 was examined in 20 cases. Somatic mutation was seen in 1 of 47 cases. Low level LOH on 17p13, in both HPV +ve and -ve cases, was found (3 of 20 cases), with or without TP53 mutation. The authors therefore suggest that somatic mutation in the hotspot regions of TP53 gene occurs infrequently in cervical cancer, and that no consistent correlation exists between TP53 mutation, LOH on 17p, and HPV status.

In 1992 Kaelbling et al. reported failure to find LOH on 17p13 or TP53 mutation in 4 HPV -ve cervical cancer cases (1.2.8.2.). The authors suspected that this could be either due to the selective use of probes for loci at 17p13.3 in that study, such that the probes failed to detect LOH involving the TP53 locus, or that the LOH found could not include TP53 at p13.1 locus. The distance between these sub-bands, p13.1-13.3, is very small that the above explanation is unlikely to be true. Failure to find LOH or mutation of TP53 in the 4 cases (above) did not alter the authors' conclusion that the loss of the normal function of TP53 is likely to be by mutation in HPV-ve cervical cancer.

Because of the known interaction of HPV oncoproteins with the TP53 gene products, and on account of the potential role of TP53 mutation in HPV -ve cervical cancer, in 1994 Mitra et al., reported a study of LOH at 17p13 (1.2.8.2.) using several markers. They also assessed the TP53 gene mutation in exon 4-9 by PCR-SSCP analysis. LOH at the TP53 locus was found in 24% of cases without mutation in exon 4-9 of the TP53 gene. All 5 tumours showed LOH at TP53 were HPV +ve. One HPV -ve tumour showed TP53 mutation. Only 11.6% of the HPV -ve cases showed mutations. Because of infrequent mutation in TP53 in HPV -ve tumours, there appeared to be no relationship between LOH at TP53 and the HPV status in the tumour samples. See Busby-Earle et al. above). Therefore, the deletion shown by the TP53 probe could alternatively be the deletion in any other tumour suppressor gene in the vicinity of TP53.
1.3. Methods of analysis

1.3.1. Conventional cytogenetics

A band is the part of a chromosome which is clearly distinguishable from its adjacent segments, by appearing darker or lighter by use of different banding techniques (Wolstenholme, 1992). The various techniques include Giemsa banding (G-banding), Quinacrine banding (Q-banding), Constitutive heterochromatin banding (C-banding), Reverse banding (R-banding), Nuclear organiser region staining (NOR), Diamino phenyl-indole fluorescent dye with DNA affinity for A+T specific binding (DAPI banding), differential replication staining with the incorporation of 5-bromodeoxyuridine (BrdU), and a number of other banding techniques (Benn and Perle, 1992).

G banding

G banding by Giemsa staining is the most widely used technique for the routine staining of mammalian chromosomes. In order to obtain this staining, the slides are treated with trypsin, and then heated in a sodium citrate & sodium chloride solution (SSC). Dark bands correlate with areas generally replicating their DNA late in S-phase, containing A+T rich DNA, and appearing to contain relatively few active genes.

Slides for G-banding are normally aged 3-5 days at room temperature, or overnight at 60°C for optimal results. Alternatively, slides can be heated at 90°C for 90 min if it is necessary to carry out the chromosome analysis on the day of harvesting (Benn and Perle 1992).
1.3.2. Fluorescence In Situ Hybridization

The concept of applying molecular hybridization directly to cytological material was initially pioneered by Gall and Pardue, (1969). During the following decade, application was restricted to highly represented sequences. It was possible to localise single sequences on metaphase chromosomes by auto-radiography of radio-isotope-labelled probes. However, this method has many limitations. The resolution is limited to relatively large chromosomal segments. In view of statistical analysis of grain distribution, 50 -100 metaphases are needed, and this is obviously time consuming. To overcome these limitations, Rudkin and Stollar, in 1977 described a non-isotopic in-situ technique using an antibody to RNA-DNA hybrids. An indirect non-isotopic technique was developed, using chemically modified DNA probes, which are detected after hybridisation by affinity reagents such as the avidin and biotin system. In the early 1980s a method for direct labelling of fluorochromes to DNA probes was developed. Fluorescein isothiocyanate (FITC) and rodamine (TRITC) are most frequently used. In order to improve the detection of the fluorescent signal, amplification of the detecting agent by an antibody stacking technique was described by Pinkel et al. in 1986 (Pinkel's sandwich) (Figure 7).

The principle of fluorescence in situ hybridization (FISH) is simple. DNA or RNA sequences are first labelled with reporter molecules. The probe and the target chromosomes or nuclei are denatured. Complementary sequences in the probes and target are then allowed to reanneal. After washing and incubation in fluorescently labelled affinity reagents, a discrete fluorescent signal is visible at the site of probe hybridization (Trask, 1991).

Application of FISH has expanded rapidly since introduction in the late 1970s (Rudkin and Stollar, 1977). It has been used in many areas of biological studies including cancer genetics, cytogenetics, pre-natal diagnosis and gene mapping (Trask, 1991). FISH has advantages over hybridization with isotopically labelled probes in spatial resolution, speed and probe stability. The sensitivity of FISH approaches that of isotopically labelled probes (Pinkel et al., 1988). A variety of probes are available for simultaneous detection in different colours, of two or more sequences in the same nucleus (Trask, 1991). DNA sequences can be
localised in targets ranging from metaphases to interphase nuclei. Indirectly labelled or directly fluorescent-labelled probes are commercially available. The whole chromosome, chromosomal regions or single-copy sequences can be detected depend on the nature of the probes used and hybridization of repetitive sequences can be suppressed by pre-hybridization of probes with unlabelled genomic DNA (Trask, 1991).

In order to obtain satisfactory results, there are three essential components in the in-situ hybridisation procedure.

1. Target sequences must be well preserved throughout hybridisation, but they should remain in an accessible state within the biological material.
2. The probe must hybridise to the target molecules with high efficiency, without substantial non-specific adherence to biological material.
3. The reporter (e.g. biotin) must bind to the probe in sufficient quantities to give a detectable signal.

Failure of any one of the components of this process results in a lower signal-to-noise ratio, and a low sensitivity for a given detection system. One-step fluorescence-avidin detection can detect a single sequence as small as 5kb by standard fluorescence microscopy (Lawrence et al., 1988).
Figure 7  Diagram of FISH Basic Principle

Pinkel et al., 1986
1.3.3. Comparative Genomic Hybridization

Introduction

Comparative Genomic Hybridization (CGH) is a powerful new method for molecular cytogenetic analysis of cancer. It is a technique for genome-scale DNA sequence copy number analysis, with the expectation of improvement in detection of DNA gains, amplifications, losses and deletions, which would be valuable in cancer research, especially for solid tumours, since relatively little is known about cytogenetic aberrations in common solid tumours as compared with the existing enormous database of detailed information on changes in haematological malignancies (Kallioniemi et al., 1992, 1993 and 1994).

CGH is particularly useful for the analysis of DNA sequence copy number changes in common solid tumours, where high-quality metaphase preparations are often difficult to make, and also where complex karyotypes are found with numerous markers. CGH provides genome-scale overviews of DNA sequence copy number changes in solid tumours, regardless of mitotic activity. It can also facilitate the analysis of the most complicated karyotypes (Kallioniemi et al., 1992, 1993 and 1994).

Genomic-scale allelic loss studies (allelotyping), for recognition of potential tumour, suppressor gene loci in solid tumours, are labour intensive. If physical deletions were to play a major role in the generation of allelic loss, a genome-wide DNA sequence copy number analysis would provide information on all potential tumour suppressor loci in a single experiment (Kallioniemi et al., 1992, 1993 and 1994).

Several studies suggest that chromosomal structures indicative of gene amplification, such as double minutes (DM) and homogeneously stained chromosomal regions (HSR), are found frequently in some solid tumours, and these structures often do contain oncogenes. Identification of the chromosomal origin of such amplified sequences could highlight locations of potentially important new oncogenes (Kallioniemi et al., 1992, 1993 and 1994).
In a single hybridization, CGH provides an overview of DNA sequence copy number changes (losses, deletions, gains & amplifications) in a tumour sample, and maps these changes onto normal chromosomes. CGH is based on the in-situ hybridization of differentially labelled total genomic tumour DNA, and of normal reference DNA on to normal human metaphase chromosomes (Figure 8). After hybridization and fluorescent staining of the bound DNA, copy number variations among the different sequences in the tumour DNA are detected by measuring the tumour : normal fluorescence intensity ratio for each locus in the target metaphase chromosomes (Kallioniemi et al., 1992, 1993 and 1994).

CGH is currently a research tool. Further technical development is required before it can be used for routine clinical applications. CGH is a supplement and not a replacement for other current technologies.
Comparative Genomic Hybridization

**Figure 8**  Diagram of Comparative Genomic Hybridization Protocol

Kallioniemi *et al.*, 1993
CGH procedure

Metaphase chromosome preparations

Normal human metaphases provide the hybridisation targets for CGH. The quality of the CGH analysis is extremely dependent on the characteristics of the metaphase spreads. Since each element of the probe is present at a very low concentration, accessibility of the target sequences to the probe is critical. Slides that are adequate for chromosome paint probes or for YACs/cosmids do not necessarily give results in CGH. Hybridisation characteristics of slides are tested using labelled DNA from normal cells (normal vs normal DNA). The reasons for variable hybridisation characteristics remain unknown. Deficient preparations are often morphologically indistinguishable from those that hybridise well. In CGH analysis, all reasonably straight, non-overlapping chromosomes are analysed at each metaphase. Data from at least 10 spreads are combined. The procedure is more efficient if the number of overlapping chromosomes is minimal. Short chromosomes are not included, as the resolution along the chromosome axis tends to be poor. DAPI is used as the counter-stain in order to obtain a faint G-banding along the chromosomes. In addition to adequate hybridisation quality, the chromosomes also need to retain their morphology so that they can be identified, and hybridisation intensity changes assigned to chromosome bands (Kallioniemi et al., 1992, 1993 and 1994).

DNA isolation

It is very important that DNA is isolated from those specimens which are histologically representative of the tumour, and which contain as high a proportion of malignant cells as possible. Any contamination of normal cells significantly dilutes the ability to see copy number changes. Since necrotic tissue and areas of inflammation may contain degraded DNA, they should be avoided. Most methods of extracting DNA from cell lines and primary tumours yield high molecular weight DNA suitable for CGH (Kallioniemi et al., 1992, 1993 and 1994).
DNA labelling

Nick translation is used for labelling genomic DNA with biotin-16-dUTP (tumour DNA) and digoxigenin-11-dUTP (normal DNA). The optimal fragment length of genomic probes after nick translation is between 600 and 2000bp. The fragment length is modified by adjusting the ratio of DNAase to DNA polymerase in the nick translation reaction. Long fragments improve the intensity and uniformity of the hybridisation, as compared to the 300 - 600 bp fragments usually obtained with a nick translation kit. Directly fluorochrome-conjugated nucleotides, such as FITC-dUTP and Texas Red-dUTP, can also be used (Kallioniemi et al., 1992, 1993 and 1994).

Hybridisation

Essentially this step follows the FISH chromosome painting procedure and that for FISH with YAC / cosmid probes (Pinkel, 1988; Kallioniemi, 1992 and 1994). For biotin and digoxigenin-labelled DNA, 120 ng is recommended, and for directly fluorochrome-conjugated DNA 200 ng are desirable for each hybridisation. 5 - 10 μg Cot-1 DNA is added to the hybridisation mix to block binding of the labelled repetitive sequences in both genomes. If repeated sequences were not adequately blocked, there would be a tendency for large ratio changes to occur at the peri-centromeric and heterochromatic regions.

To achieve the required DNA concentration, the labelled DNA and blocking DNA are mixed, precipitated by ethanol, and dissolved in the hybridization buffer. Before hybridisation, the slides are digested by use of proteinase K, and denatured at 72-74°C for 3 minutes in 70% formamide / 2xSSC. Denaturation time, temperature and the amount of proteinase K digestion required vary from one batch of slides to another. The aim is to treat the slides as vigorously as possible to maximise denaturation and to maximise probe penetration, but to avoid destroying the DAPI banding pattern necessary for chromosome identification. Hybridisation is carried out under the cover-slip in a moist chamber at 37°C for 2 days. Washing of unbound probes is carried out as in the FISH protocols as described by Pinkel et al., 1988 and by Kallioneimi et al., 1992 and 1994. For indirectly
labelled probes, immunochemical detection with avidin-FITC (green fluorescence for tumour DNA) and anti-digoxigenin rhodamine (red fluorescence for normal DNA) is carried out. Only one layer of each detection is used since this results in the highest signal: noise ratio and the most uniform signal (Kallioniemi et al., 1992, 1993 and 1994).
Fluorescence microscopy and image analysis

a. Visual analysis

CGH analysis is dependent on the evaluation of the relative intensities of the two fluorochromes. This can be accomplished either by computer-assisted multicolour image analysis or by visual multicolour fluorescence microscopy. For visual analysis, the intensity of two fluorescent dyes must be compared along the length of a chromosome. This requires the use of double or triple band pass filters which allow simultaneous visualisation of the two fluorochromes.

High level gene amplifications (> 10 - 20 fold), as well as changes affecting large regions, such as gains and losses of entire chromosome arms, are detectable visually in homogeneous cell lines.

However, small deletions and other lower level copy number differences often cannot be detected reliably because of the limited ability of the human eye to detect small variations in colour balance.

Because of the genetic heterogeneity and normal cell contamination, ratio changes found in the analysis of primary tumours often cannot be evaluated reliably without digital imaging (Kallioniemi et al., 1992, 1993 and 1994).

b. Digital image analysis

The use of digital image analysis improves both the quantitation and the display of CGH results. The analysis procedure is based on both the visual evaluation of digital images and a quantitative analysis of fluorescence ratios.

It is necessary first to scan carefully through each entire slide to find the best metaphases. Images of 5 or more metaphases of the highest quality are collected from each slide. Images are acquired using a multicolour quantitative image processing system (QUIPS), which is superimposed upon a regular fluorescence microscope (Nikon / Zeiss Axiophot) equipped with a high resolution cooled or non-cooled CCD camera and triple band pass beam splitter and emission filters (Kallioniemi et al., 1992, 1993 and 1994).
Grey level images of each of the fluorochromes are obtained using a computer-controlled filter-wheel to insert single-band-pass excitation filters to excite sequentially DAPI, FITC and rhodamine. This allows visualisation of all three fluorochromes without any registration shifts between images, and with negligible cross-talk between the fluorochromes. Exposure times for each colour are adjusted to obtain high intensity without saturation.

As CGH requires acquisition of high-resolution images from the entire metaphase, the resulting images can be composed of up to 1 million pixels. For visual assessment, the three single-colour images are overlaid and displayed in pseudo colours matching approximately the original colours of the fluorochromes and counter stain used. By display adjustments, the relative intensities of green and red fluorescence are balanced and any small colour ratio differences made more readily visible through the use of contrast-stretching. Chromosomes are identified using a technique based on a contrast-stretched image of the DAPI counter stain. The three fluorochrome-specific images obtained from each metaphase with the CCD camera are stored for subsequent analysis.

Quantitative analysis of green to red fluorescence ratios along metaphase chromosomes gives the most objective assessment of copy number changes. Quantitative ratio analysis is done using the program based on chromosome image analysis algorithms (Kallioniemi et al., 1994).

The chromosomes are first segmented, in a manner based on the combined DAPI and red (normal DNA) images. In order to be able to compare ratios between different metaphases, and between different slides or hybridisation, it is necessary to standardise the green and red fluorescence intensities of each metaphase, so that the effects of differences in the hybridisation and imaging conditions between separate experiments are minimised. Global background is subtracted in order to normalise the green and red fluorescence intensities, so that the average normalised green : red intensity ratio is 1.0 for the entire metaphase.

Errors in the segmentation of chromosomes are corrected by separation of touching chromosomes. Chromosomes previously identified by DAPI banding, are selected one at a
time from the metaphase. The software measures the background intensities of each fluorochrome in the immediate vicinity of each chromosome. The background level is defined as a set of pixels surrounding the chromosome of interest, along a path of strictly decreasing fluorescence intensity. The medial axis of the chromosome is determined and displayed. Green and red fluorescence intensities, corrected by subtracting the local background, and then multiplied by the intensity scaling factors, are integrated along lines that are perpendicular to the medial axis, and are spaced at one pixel intervals along the axis. The integration covers both chromatids, and comprises the sum of two independent observations. The integrated green and red fluorescence ratios that run from pter to qter of each chromosome are then used to calculate the green:red ratio profile for each chromosome.

Random noise in hybridisation limits the ability to recognise copy number changes. CGH signals on chromosome homologues in each metaphase spread should be identical. Therefore data from multiple metaphases is combined, so as to reduce the noise and to enhance the visibility of real copy number changes. Data from many chromosomes from 10-20 metaphases are used, and profiles of the mean ratio and one SD are calculated.

Accurate implementation of profile averaging is difficult because the same chromosomes may be differently condensed. Condensation may be not uniform along the length of each chromosome. Different condensation is overcome by normalising all ratio profiles to a standard length for that chromosome type. However, if the condensation is not uniform, ratio changes may occur at slightly different locations along the medial axis of each chromosome. Therefore, the combination of profiles from several chromosomes may obscure the observations, especially those affecting small regions.
CGH guidelines

CGH images

Detecting copy number changes in a genome using CGH is critically dependent on the quality of the hybridisation. Combination of data from numerous metaphases, and the advanced technology of image analysis, cannot compensate for poor quality hybridisation. Criteria for adequate quality for quantitative ratio profile analysis are as follows:

a). Metaphase spreads should show smooth high intensity hybridisation. Since granularity gives increased noise, metaphase spreads with non-specific granules and fluorescent spots on the chromosomes are not analysed. Inadequate denaturation of target DNA could lead to a prominent variation in hybridisation intensities of both colours along chromosomes, which might resemble a banding pattern, and analysis of such a metaphase could therefore be unreliable.

b). Green and red fluorescence distributions are similar to those observed in the two sister chromatids of a chromosome, in the two chromosome homologues in each metaphase, and in the same chromosomes in different metaphases.

c). The background fluorescence level on the slide surrounding the chromosomes must be low and uniform.

d). Binding of the labelled DNAs to centromeric regions and heterochromatic regions must be negligible.

e). Chromosomes must have good DAPI bands, adequate chromosome length, and minimal overlapping.

Quality control

CGH experiments with two normal DNA samples can be used as a negative control. Therefore, only ratio changes that exceed the fluctuation seen in the negative control are interpreted as evidence of real DNA gain or loss in tumour samples. In good quality hybridisation, normal variation, with one SD each side of the mean, should be within the ratio of 0.85 - 1.15. Short arms of acrocentric chromosomes, telomeric regions, peri-centric
and heterochromatic regions fall outside this range, because of low signal intensities, and these regions are never included in an analysis (Kallioniemi et al., 1992, 1993 and 1994).

**Data interpretation**

Ratio imaging provides the most accurate and objective way to assess copy number change. The consistency of the green : red ratio from one metaphase to another can be used to judge the quality of hybridisation. If the resulting ratio falls outside one standard deviation on either side of the mean, the results should not be used. Ratio profiles are the outcome of the computerised digital image analysis system, and are used for the comparison with the visual assessment.

The quantitative linear relation between the DNA sequence copy number and the green:red ratio is only obtained when the size of the region affected is large enough (>10-20Mb). However, qualitative detection of copy number changes affecting smaller regions (a few hundred Kb) is possible if the DNA sequences show high level amplification. The total amount of amplified DNA = amplicon size x the level of amplification. The total amount of amplified DNA must be at least 2 Mb for detection by CGH to be possible.

Detection of 10 - 20 Mb or larger deletions is possible, but it is more difficult to detect a loss of one chromosome homologue in a tetraploid cell line than in a diploid one. In the tetraploid cell line, the green to red ratio is expected to change by only 25%, from four to three copies, as compared to 50% in the diploid line, from two to one copy. An accuracy of 3 - 5 Mb may be achieved by CGH, but this may be limited by the effect of denaturation on chromosome morphology. After hybridisation, a DAPI banded chromosome will only show a 10 - 20 Mb resolution (Kallioniemi et al., 1992, 1993 and 1994).
Advantages of CGH

Amplification in the genome can be detected without any specific probes or any prior information of the amplified loci. Therefore it is an ideal method for rapid "screening", and can also map the amplification regions on normal chromosomes.

The amplification observed by CGH at previously unsuspected loci can be a starting point for the isolation of actual target genes Kallioniemi et al, 1992, 1993 and 1994).

Disadvantages of CGH

CGH can only detect genetic abnormality that involves loss or gain of DNA sequences. Therefore, balanced translocations and inversions are not detectable.

CGH can detect DNA sequence copy number changes relative to the average number in the whole tumour sample. Therefore it cannot distinguish diploid tumours from true triploid or tetraploid specimens as no differences in the green:red ratio between or within chromosomes can be detected.

CGH cannot reliably analyse DNA in the pericentric and heterochromatic regions, as these are blocked by unlabelled Cot-1 DNA in the hybridisation process. Also DNA sequences in these regions are highly polymorphic in copy number between individuals. It is important to be cautious in the interpretation of ratio changes at telomeres, since the green and red fluorescence intensities gradually decrease at the telomeres, approaching the levels of background fluorescence, and ratio changes are unreliable.

CGH detects the average copy number of sequences in all cells in the tumour samples. Therefore genetic changes that are present homogeneously in the tumour cells are more readily detected. However, in multiple clonal tumours, different genetic changes present in individual clones may sometimes balance one another, and are found at too low a frequency to be detected.
1p32-pter, 16p, 19 & 22 may occasionally be found to be below the average ratio and this can lead to false positive interpretation of a deletion. This is due to the differential hybridisation properties of digoxigenin and biotin-labelled probes. Other chromosomal regions do not seem to be so affected, and false positive gains of DNA sequences have not been seen at any locus (Kallioniemi et al., 1992, 1993 and 1994).
IV. Aims of the present study

The aim was to investigate genetic changes in cultured cervical tumour material using molecular cytogenetic techniques in order to get some idea which changes might be important in the initiation and development of cervical cancer and to identify the potential locations of genes predisposing to cervical cancer.

Cell lines were used in order to obtain large numbers of good quality metaphases to investigate the chromosome abnormalities in detail. It is well known that it is quite difficult to obtain good quality metaphases from direct preparations from tumour samples.

Conventional G banding was used to obtain the background karyotypes of all cultured cervical cancer cell lines.

FISH chromosome painting was used to investigate every individual chromosome to clarify the complex chromosome rearrangements in the karyotypes and YAC and cosmid probes were used to identify and characterise the breakpoints for each cell line. These could then be compared in the five lines from three different histological groups to see whether any were non-random and/or confined to a particular histological type.

Comparative Genomic Hybridization (CGH) was applied using the DNA extracted from three of the cell lines already studied by FISH to test the sensitivity and reproducibility of this technique.

CGH was also applied using the DNA extracted from an uncultured fresh cervical squamous tumour sample to see how in-vivo changes compared with those found in cultured material, and to assess which changes might be important in the initiation and development of cervical cancer.

CGH was used to identify the chromosomal origin of amplified sequences, which could highlight the locations of potentially important new oncogenes.
Chapter 2

Materials and Methods
Materials and Methods

2.1. Materials

2.1.1. Cell lines

Five newly established cell lines, three derived from squamous carcinoma of the cervix (SM7, EH2 & DE3), one from an adenocarcinoma of the cervix (JE6), and one from an adeno-squamous carcinoma (XH1) were studied. All of these were set up as described below (Han et al., 1991) and supplied by Dr. Xin Han, St.Thomas' Hospital, London.

All cell lines were established in long-term culture and grown as flat adherent monolayers of polygonal cells. They were all studied at low passage number, mostly <20.

a) Background clinical history

SM7
This cell line originated from a 42 year old lady, who presented with a heavy, foul smelling discharge and post-coital bleeding. The preoperative clinical stage was Ib and a Wertheim's hysterectomy was performed. Histology showed a poorly differentiated squamous carcinoma with upward extension to the isthmus. Postoperative re-staging indicated IIA and four cycles of chemotherapy were given, but the tumour recurred in the pelvis ten months afterwards. Radiotherapy was administered locally to the pelvis with an additional four courses of a single agent chemotherapy, but unfortunately the patient died six months after the recurrence. SM7 was established from the cervical tumour from the Wertheim's hysterectomy specimen.

EH2
This cell line originated from a 57 year old lady who presented with post-menopausal bleeding and also with a history of an abnormal smear of CIN II. A colposcopic biopsy of the cervix revealed CIN III with features of invasion. A pre-operative staging of IIA was indicated. She underwent Wertheim's hysterectomy. The histology agreed with a stage IIA and post-operative radiotherapy was given. EH2 was established from the Wertheim's hysterectomy specimen. The patient attended the follow up clinic last year.
DE3
This line originated from a 40 year old lady who presented with a watery vaginal discharge and post coital bleeding. Clinical staging was II and the biopsy histology showed a squamous cell carcinoma. Both internal catheter insertion and external radiation were given. Two and a half years later, local pelvic recurrence occurred and a tumour and pelvic organs were surgically removed. Three years later the pelvic tumour recurred for a second time and an anterior pelvic exenteration was performed. Histology showed a moderately differentiated invasive squamous cell carcinoma. DE3 was established from the second recurrence of the tumour from the anterior exenteration specimen which occurred five and a half years after the primary tumour.

JE6
This cell line originated from a 62 year old lady who presented with post-menopausal bleeding. A biopsy was taken from the tumour mass at the vaginal vault and revealed a poorly differentiated adenocarcinoma of the cervix. This patient had previously had a subtotal hysterectomy and bilateral oophorectomy 20 years before, probably for an ovarian tumour, but information regarding that episode was quite sparse since it happened in a different hospital and it was impossible to find the records of 20 years back. An exenteration operation, radiotherapy and chemotherapy were given for the cervical tumour but the patient died after 6 months. JE6 was established from the pelvic exenteration specimen of the cervical tumour.

XH1
This cell line originated from a 32 year old lady who presented with post coital bleeding and irregular vaginal bleeding. Pre-operative clinical staging was Ib and she underwent a Wertheim's hysterectomy. Histology showed a moderately differentiated keratinising adenosquamous carcinoma and stage Ia was confirmed. Post-operative radiotherapy was applied to the pelvis but pelvic recurrence occurred 9 months after the operation. Chemotherapy was given but she died 3 months later. XH1 was established from the Wertheim's hysterectomy specimen.

The clinical history and histology of the tumours is summarised in table 2.
Table 2

Clinical background of the cell lines

<table>
<thead>
<tr>
<th>cell lines</th>
<th>age</th>
<th>FIGO</th>
<th>Operation</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM7</td>
<td>42</td>
<td>IIa</td>
<td>Wertheim's hysterectomy</td>
<td>poorly differentiated squamous cell carcinoma</td>
</tr>
<tr>
<td>EH2</td>
<td>57</td>
<td>IIa</td>
<td>Wertheim's hysterectomy</td>
<td>moderate to poorly differentiated focally keratinizing squamous cell carcinoma</td>
</tr>
<tr>
<td>DE3</td>
<td>40</td>
<td>IIb</td>
<td>Pelvic exenteration</td>
<td>moderately differentiated keratinizing squamous cell carcinoma</td>
</tr>
<tr>
<td>JE6</td>
<td>62</td>
<td>III</td>
<td>Pelvic exenteration</td>
<td>moderate to poorly differentiated adenocarcinoma</td>
</tr>
<tr>
<td>XH1</td>
<td>32</td>
<td>Ib</td>
<td>Wertheim's hysterectomy</td>
<td>moderately differentiated focally keratinizing adeno-squamous ca</td>
</tr>
</tbody>
</table>
b) DNA profile analysis has demonstrated that all lines were developed from different individuals without cross contamination (Han et al., 1991).

c) HPV status in cell lines
The human papilloma virus (HPV) infection status in all lines was determined by Dr Xin Han using the polymerase chain reaction (PCR) and the results are as shown in table 3 below.

Table 3.

<table>
<thead>
<tr>
<th>HPV status in cervical cancer cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
</tr>
<tr>
<td>SM7</td>
</tr>
<tr>
<td>EH2</td>
</tr>
<tr>
<td>DE3</td>
</tr>
<tr>
<td>JE6</td>
</tr>
<tr>
<td>XH1</td>
</tr>
</tbody>
</table>

2.1.2. Primary fresh cervical tumour sample
This was obtained from a Wertheim's heterectomy operative sample, taken from a 56 year old lady with stage II\textsubscript{c} cervical cancer. Histology was a poorly differentiated squamous carcinoma.

2.1.3. Total human chromosome specific paint probes (Paints)
All 23 chromosome paints (1 - 22 & X) used in this study were supplied already biotin labelled by Cambio.
2.1.4. Yeast Artificial Chromosomes (YACs)

YACs with human DNA sequences cloned from 3p and 3q, mapped in our laboratory for the HGMP project (Professor S Povey et al., Galton laboratory UCL) were used. All YACs used in this study are listed in table 4.

<table>
<thead>
<tr>
<th>Probe number</th>
<th>Type</th>
<th>Location</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>262</td>
<td>YAC</td>
<td>3p21</td>
<td>HGMP resource centre</td>
</tr>
<tr>
<td>36</td>
<td>YAC</td>
<td>3p25-26</td>
<td>&quot;</td>
</tr>
<tr>
<td>303</td>
<td>YAC</td>
<td>3q21</td>
<td>&quot;</td>
</tr>
<tr>
<td>168</td>
<td>YAC</td>
<td>3q24-26</td>
<td>&quot;</td>
</tr>
</tbody>
</table>
2.1.5. Cosmids

Cosmids containing human DNA sequences from chromosome 3p and 3q were provided by Dr. K. Hashimoto and Dr. Y. Nakamura from the Japanese Cancer Research Resources Bank (JCRB) - Gene, National Institute of Health Tokyo, Japan.

The cosmid cloned from chromosome 9q34 was provided by Dr. Karen Woodward (formerly at the Galton laboratory) and originated from the Lawrence Livermore chromosome 9 flow specific cosmid library.

All cosmid probes used in this study and their locations are shown in table 5.

### Table 5.

<table>
<thead>
<tr>
<th>Probe number</th>
<th>Type</th>
<th>Location</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3S939</td>
<td>Cosmid</td>
<td>3q27</td>
<td>&quot;</td>
</tr>
<tr>
<td>256C3</td>
<td>Cosmid</td>
<td>9q34</td>
<td>Lawrence Livermore National Laboratory</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chromosome 9 specific library</td>
</tr>
</tbody>
</table>
2.1.6. General standard solutions and buffers

Solutions and buffers were prepared using distilled de-ionised water and were stored at room temperature, 15 - 25°C, and sterilised by autoclaving at 15lbs per square inch at 121°C for 20 minutes. The components for standard solutions and buffers are shown in Appendix A.

2.1.7. Chemicals

Chemicals for general use (analar quality) were obtained from British Drug House (BDH), Poole, Dorset, unless otherwise stated. Glycerol was also obtained from BDH. Tween 20 detergent and phosphate buffered saline (PBS) tablets were from the Sigma and Dextran sulphate from Pharmacia.

2.1.8. Enzymes

Proteinase K (Boehringer Mannheim) was stored as a stock solution (50μg/ml), at -20°C, (Appendix F), and a final working concentration of 0.035 μg/ml with PK buffer was used for the pre-hybridisation treatment of slides. A final concentration of 10mg / ml was used for DNA extraction. Lyticase (Sigma) was made up at a final concentration of 25U/μl in sterile water and stored at 4°C. Pancreatic RNAse A (Sigma) was made up at a concentration of 10mg/ml in 2 x SSC, boiled for 10 minutes and stored at -20°C.

2.1.9. Cell culture media materials and solutions

Dulbecco's Minimal Essential Medium (DMEM), non essential aminoacids and glutamine were obtained from Gibco BRL, fetal calf serum, penicillin 10,000 U and streptomycin (10 mg in 0.9% saline) from Sigma, and the 50ml cell culture flasks were from Nunclon. Components of the media are shown in Appendix B.
2.1.10. **G banding materials and solutions**

Giemsa staining solution and Sorensen's buffer solution / Gurr buffer tablets were obtained from BDH and 5% Bacto trypsin (1:250) from Difco laboratories. Components for banding are shown in Appendix C.

2.1.11. **Yeast Artificial chromosome (YAC) culture media materials and solutions**

Bacto-yeast nitrogen base without amino acids, bacto-agar, casamino acids came from Difco, Lyticase and β-mercapto-ethanol from Sigma, and glycerol, glucose and sucrose from BDH. Components of yeast culture media are shown in Appendix D.

2.1.12. **Cosmid culture media material and solutions**

Tryptone and yeast extract were obtained from Difco, and Ampicillin from Sigma. Components of the cosmid culture media are shown in Appendix E.

2.1.13. **Fluorescence In Situ Hybridization materials and solutions**

All solutions for fluorescence in situ hybridisation were prepared using distilled deionised water and stored at room temperature. The biotin labelling kit (BioNick) was provided by GibcoBRL and the Nick Translation kit and digoxigenin-11dUTP by Boehringer Mannheim. Fluorescein avidin and biotinylated anti-avidin D came from Vector, mouse anti-digoxigenin, TRITC conjugated rabbit anti-mouse antibody, TRITC conjugated goat anti-rabbit antibody from the Sigma, and Cot 1 DNA was from GibcoBRL. Components for solutions for FISH are shown in Appendix F.

2.1.14. **Comparative Genomic Hybridization materials and solutions**

All solutions and buffers for Comparative Genomic Hybridization have already been described in 2.1.13. and 2.1.6–8. (Appendix A, F & G).
2.2. Methods

2.2.1. Cell line cultures

The initiation, DNA testing and HPV analysis on these cell lines was done by Dr. Xin Han of the histopathology department at St Thomas' Hospital, London.

2.2.1.1.a. Initiation of long term cultures (Han et al. 1991)

Cell lines were established from operative specimens, three from Wertheim's hysterectomy and two from pelvic exenteration as shown in Table 1. Portions of the tumours were minced and incubated in protease (Sigma Chemical Co, Dorset), collagenase (Lorne Diagnostics Ltd, Suffolk) and DNAse (Sigma) in EBSS (Earle's Balanced Salt Solution) containing 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL Ltd, Middx). Single cells and small clumps were seeded into a flask containing Mitomycin-C treated Swiss albino embryonic mouse fibroblast 3T3 cells (ATCC CCL92) (ICN Flow Lab Ltd, Bucks) as a feeder layer. An enriched medium was used consisting of DMEM (ICN Flow) 20% fetal calf serum, 2mM L-glutamine, antibiotics, 0.4 µg/ml hydrocortisone (Glaxo), 10 µg/ml bovine insulin (Sigma), 10 ng/ml epidermal growth factor (ICN Flow) and 10 ng/ml cholera toxin (Sigma). At passage 4, a colony consisting predominantly of small rapidly dividing epithelial cells was selected and passaged further. Fibroblasts from the cervical stroma were removed by incubation with 0.02% EDTA in EBSS at 37°C for 2-3 mins and then by vigorous pipetting against the surface of the flask. The 3T3 feeder layer was omitted from passage 6 onwards.

b) Testing for cross contamination

DNA samples from these cell lines were utilised in DNA profile analysis, (Han et al. 1991), to demonstrate that the lines were developed from different individuals without cross contamination. Briefly, DNA samples were digested with Hinf I and electrophoresed. The resulting DNA fragments were denatured, transferred and hybridised with the hypervariable minisatellite DNA probe, MS31, labelled with digoxigenin (BCL).
c) HPV status

Total cellular DNA was purified by lysing the cultured cells in 10mM Tris-Cl (pH 8), 10mM NaCl, 10mM EDTA 0.5%, SDS containing 100 μg/ml proteinase K, followed by protein extraction using phenol. RNA was digested by RNase. The human papilloma virus (HPV) infection status was determined by the polymerase chain reaction (PCR). DNA amplification was performed firstly using the primers MY09 and MY11, which led to amplification of a region located in the L1 open reading frame of all sequenced HPVs. Type specific primers for HPV 6, 11, 16, 18, 31 and 33 were subsequently used to determined the HPV type in the positive cell lines (Han et al 1991).

2.2.1.2. Culture maintenance

The medium consisted of DMEM with 10% fetal calf serum, 2 ml GPS and supplements of 400 μl hydrocortisone, 50 μl epithelial growth factor and 40 μl insulin were added to 100 ml of medium. Cultures were placed in the 8% CO₂ incubator at 37°C and the growth monitored under the inverted microscope every day. The medium was changed twice a week until the cultures were ready to trypsinise and then changed 24 hrs before the harvest.

2.2.1.3. Subculture (Trypsinization)

The medium was removed from the culture and the cells washed in Hank's balanced salt solution. The monolayer was soaked in a versene solution to loosen the cells. The versene was removed, trypsin in versene was added and incubated at 37°C for 5-10 min. The flask was tapped to dislodge the cells and the culture checked under the microscope to see whether any cells were still attached. When almost all the cells had been removed, equal volumes were transferred into 2 or 3 new flasks depending on the density of the cellular monolayer. Then 5 ml of new medium was added to each new 25 cm² (T25) flask. The tops were loosened and incubated at 37°C in 8% CO₂. Cultures were trypsinised 2 to 3 days prior to harvest.
2.2.1.4. Harvesting for metaphase chromosomes

Twenty to thirty microlitre of colcemid (10μg/ml) was added to 5 ml of medium and incubated at 37°C for 1 - 3 hrs. The medium was removed and placed in a centrifuge tube. The monolayer cells were washed in Hank's solution, soaked in versene and incubated in trypsin and versene. All washings and ultimately the cell suspension were added to the centrifuge tube and centrifuged at 1000rpm for 5 mins. The supernatant was discarded and the cells re-suspended in 0.075M hypotonic KCL then incubated at 37°C for 30 mins. The tubes were centrifuged again at 1000rpm for 5 mins, and the supernatant discarded. Then the pellet was re-suspended in 3:1 methanol : glacial acetic acid fixative which was added dropwise, tapping the tube vigorously in between the first few drops to obtain an even suspension, until the tube was filled to 1/2 or 1/3 depending on the size of the pellet. The cell suspension was stored at 4°C overnight or until slides were made.

2.2.1.5. Slide preparation

Prior to adding the cell suspension the slides were soaked in methanol with a few drops of conc HCL. They were dried immediately prior to use with a lint-free cloth. The fixed cell suspension prepared before and stored at least overnight was centrifuged at 1000 rpm for 6 mins, the supernatant discarded and the cells resuspended in fresh fixative. The fixative was changed several times in this way and then cells were re-suspended in enough fresh fixative to achieve the right concentration. Extra moisture was added to the slides by gently breathing on them. A drop of the suspension was then dropped onto the slide from a height of about 12 cm. The slide was rapidly air dried and then flooded with 70% acetic acid, left for few seconds and then air dried. Slides were checked under phase contrast microscopy to ensure that the cell density was correct, and that there were enough well spread, clean, cytoplasm free mitoses.
2.2.2. G banding

Slides were incubated in 2xSSC at 60°C for 2 hrs, rinsed briefly in buffer solution (phosphate buffer solution, pH 6.8 buffer tablets (BDH), and placed in 5% Difco bacto trypsin (1:250), 1 ml was diluted in 50 ml buffer, for 10 seconds at 10°C. Slides were rinsed in buffer again before being stained with a 4% solution of Giemsa (BDH, Gurr) in 6.8 buffer for 5 mins. They were then rinsed under running tap water and a cover slip applied to assess the quality of banding and the stain intensity under the microscope. Once the quality of slides were satisfactory, dried slides were then viewed again under the microscope, this time, under oil without a coverslip. Twenty to thirty nicely spread metaphases with long chromosomes, but without overlaps were chosen and photographs taken using Kodak TP 2415 monochrome film and the camera set at ASA 50 - 100, DIN 18 - 20 with a manual shutter speed of 0.5 - 1 sec. At least 10 metaphase spreads with good quality banding were chosen from the photographic prints. Chromosomes were cut, paired and preliminary karyotypes were established prior to the Fluorescence in situ hybridization chromosome painting work. Chromosomes which could not be identified with certainty were grouped separately under 'markers'. Then the karyotypes could be re-evaluated after chromosome painting and the breakpoints mapped.
2.2.3. Fluorescence In Situ Hybridization chromosome painting

2.2.3.1. Chromosome preparations
Metaphase spreads for FISH were prepared as described for G-banding.

2.2.3.2. Pre-hybridization slide preparation
Slides were dehydrated in an alcohol series, air dried, fixed in formaldehyde for 10 mins at room temperature, washed in PBS for 5 mins on a stirrer, dehydrated again and air dried.

2.2.3.3. Single colour FISH painting

2.2.3.3.1. Pre-labelled DNA paint probes
A total of 23 biotin labelled human chromosome specific paint probes (1 - 22 & X) were used in this study. They were supplied with the appropriate amount of competitor DNA.

2.2.3.3.2. Hybridization
10 μl of the chromosome paint probe was applied to the slide under a coverslip and sealed with cow-gum. Both chromosome DNA and probe DNA were denatured simultaneously by incubating in an oven at 80°C for 3 mins, then allowed to hybridize overnight in a moist chamber in an incubator at 37°C.

2.2.3.3.3. Post-hybridization washes
Following hybridization, the cow gum was removed and the coverslips were soaked in 50% formamide / 2xSSC at 45°C until they came off. The washing was continued in 50% formamide / 2xSSC for 15 mins including the initial soaking. In preparation for signal detection with biotin, slides were washed for 5 mins at room temperature with SSCT (4 x SSC / 0.05% Tween 20) followed by a 20 min incubation in SSCM (4 x SSC / 5% Marvel non-fat dried milk).
2.2.3.3.4. Single colour paint signal detection

The slides were then treated as follows:

1) 20 min incubation in the dark at room temperature with avidine - fluorescein isothiocyanate (avidin - FITC) in SSCM (1:200 and 100μl volume for each slide), followed by three 5 min washes in SSCT at room temperature.

2) 20 min incubation in biotinylated - anti - avidin in SSCM (1:100 and 100μl volume for each slide) under cover at room temperature, followed by three 5 min washes at room temperature.

3) 20 min incubation under cover at room temperature with avidin - FITC in SSCM (as above) followed by one wash in SSCT at room temperature and washed twice in PBS for 5 min each. Finally, the chromosomes were counter stained by the addition of 10 μl anti-fade containing DAPI and propidium iodide (Appendix F).

2.2.3.4. Dual colour FISH paint

2.2.3.4.1. Combined use of two differently pre-labelled DNA paint probes

This involved the selective use of biotin-labelled paints simultaneously with selective FITC-labelled paints to clarify the chromosomes of origin in complex rearrangements. Biotin-labelled paints were detected with texas red and visualised as a red signal. FITC-labelled paint probe was visualised as green signals.

2.2.3.4.2. Hybridization

10 μl of biotin-labelled paint with 10 μl of FITC-labelled paint were denatured by incubating at 75 °C for 5 min and floated in a waterbath at 37°C for 15 min to pre-anneal.

Slides prepared as in 2.2.3.2. were denatured by incubating in the oven at 75°C for 3 min with 100μl of 70% v/v deionised formamide in 2 x SSC under coverslips. Slides were then dehydrated in 70% ice cold ethanol followed by 90% and 100% ethanol.

After slides were air dried, a total of 20μl of the combined differently labelled probes were applied to the slide under a coverslip and sealed with cow-gum.

Overnight hybridization was allowed to take place in a moist chamber at 37°C.
2.2.3.4.3. Post-hybridization washes and signal detection by Cambio dual-colour painting kit

Slides were washed twice by incubating for 5 min in 50% (v/v) formamide / 0.5 x SSC (1:1) at 42°C, followed by two washes in 2 x SSC for 5 min each at 42°C. Then the following steps were carried out:-

1) Detection reagent B3 was applied, diluted in blocking protein (1:500), 100 µl per slide, under the coverslip, incubated at 37°C for 20 min in a moist chamber, and followed by three washes at 42°C in 4 x SSC (500 ml) with (250 µl) detergent.

2) A mixed detection reagent B3/F1 was applied, diluted in blocking protein (1:250 & 1:400 respectively), 100 µl per slide, incubated and washed as above.

3) A mixed detection reagent B3/F2 was applied, diluted in blocking agent (1:500 & 1:100 respectively), 100 µl per slide, incubated and washed as above.

Briefly, dehydrated and air-dried slides were mounted in a mountant/counterstain mixture under the cover slip.

2.2.3.5. Image analysis

Chromosomes were visualised using a Nikon Optiphot Fluorescence microscope. Single FISH signals were visualised through an FITC filter (DM 510) and double FISH signals were visualised through a FITC/TRITC filter for simultaneous detection of FITC and TRITC. Images were analysed and stored using a Bio-Rad MRC 600 confocal laser microscope attachment with MRC 500/600 software.
1) Nikon Optiphot fluorescence microscopy

2) Filters (FITC DM510 or FITC/TRITC)

3) Bio-Rad MRC -600 laser scanning confocal imaging system

4) Mercury lamp

5) Computer image analysis system with MRC500/600 software

6) Mitsubishi polaroid colour photograph processor
2.2.4. FISH on G banded metaphases
Giemsa stain and Euparol, used for photography, were removed by a series of washes; 1 minute in xylol, 1 min in xylol/ethanol (1/1 v/v) and 10 min in methanol/ acetic acid (3/1 v/v). Air-dried slides were fixed for 10 minutes in 3.7% formaldehyde/ phosphate buffer saline (PBS), which was prepared from 37% formaldehyde stock solution (Sigma). This was followed by two 5 min washes in PBS. Hybridization, denaturation, and post hybridization washes and signal detection were the same as described above.

2.2.5. Reprobing of DNA probes on previously painted human chromosomes
Slides after in situ hybridization can be reprobed with high reliability after single or double target probing. After photography of the first probing, coverslips were carefully removed and slides are soaked in 0.1% Tween 20 in 4 x SSC (20 x 0.3 M NaCl, 0.03M NaCitrate) for 30 minutes, followed by two further 5 minute washes in 2 x SSC to remove mountant, most probes and detection agent. After dehydration through an alcohol series, the slides were covered with hybridization solution containing the new probes, re-denatured, hybridized overnight followed by washes and the signal detected according to the standard protocol. Minor degradation in chromosome morphology and occasional chromosome loss were evident after the second probing.
2.2.6. Yeast Artificial Chromosomes (YAC)

2.2.6.1. Preparation of YAC probes for FISH

2.2.6.1.1. Culture

A single YAC colony was inoculated into 10 ml SD medium (Bacto yeast Nitrogen base 7g/L, Glucose 20g/L, Adenine & Tyrosine 55mg/L) with 1 μl ampicillin (100mg/ml). The culture was incubated overnight at 30°C with agitation.

2.2.6.1.2. Extraction of human DNA from YAC culture

The culture was centrifuged at 1000 rpm for 10 min, the supernatant discarded and the pellet re-suspended in 0.5 ml YRB (1.2M Sorbitol, 10mM Tris pH 7.5, 20mM EDTA) with B mercaptoethanol 14mM 1:1000 dilution followed by 25U/μl and incubated at 37°C for 1 hour. The solution was transferred to an eppendorf tube and centrifuged at 14000 rpm for 1 min. Then the pellet was re-suspended in 500μl 50mM Tris, 20mM EDTA pH 7.4. 50μl 10% SDS was added and the tube incubated at 65°C for 30 mins. Then 200μl 5M potassium acetate was added and the tube stood on ice for 1 hour, then spun in a microcentrifuge at 14000 rpm for 5 min before transferring the supernatant to a fresh eppendorf tube and adding an equal volume of isopropanol at room temperature. This was mixed gently and allowed to settle at room temperature for 5 min before microcentrifuging at high speed for 10 sec. The supernatant was discarded and the pellet was freeze dried, then re-suspended in TE (10mM Tris, 1mM EDTA pH 7.4). 15 μl of 1 mg/ml RNase was then added and incubated at 37°C for 30 min. Thirty micro-litre of 3M Na acetate was added and phenol and chloroform extraction performed. DNA was precipitated with 100% ethanol and dissolved in TE. The extracted DNA was quantified by means of a fluorometer.
2.2.6.2. **Single colour YAC FISH**

2.2.6.2.1. Single colour YAC DNA probe labelling with the BioNick Kit

The BioNick labelling system is designed for generating small, <500 bp, biotin labelled DNA probes by nick translation. To 1 µg DNA, was added 10 x dNTP mix (0.2 mM dCTP, dGTP, dTTP, 0.1 mM dATP, 0.1mM biotin-14-dATP, 500mM pH7.8 Tris-HCl, 50 mM MgCl2, 100mM β-mercaptoethanol & 100µg/ml nuclease-free BSA) and 10 x enzyme mix (0.5 units/µl DNA Polymerase I, 0.0075 units/µl DNase I, pH 7.5 50mM Tris-HCl, 5mM magnesium acetate, 1mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 50% v/v glycerol & 100µg/ml nuclease-free BSA) and was made up to 50µl with distilled water and incubated for 1 hour at 16°C. Stop buffer, 5 µl, was added and unincorporated nucleotides were separated from the labelled DNA probe using a column.

2.2.6.2.2. Pre-hybridization preparation of labelled YAC probes

Prior to hybridization, 200ng of labelled DNA probe (in 11 µl vol) was mixed with Cot-1-DNA 10µl, 5 µl of 10mg/ml salmon sperm DNA and one twentieth the volume of 3M sodium acetate. DNA was then precipitated by the addition of two and a half volumes of absolute ethanol (99.7 - 100%) followed by 1 hour at -70°C. DNA was then retrieved by centrifugation at 14,000 g for 15 mins, and removing the supernatant and freeze dried pellet of DNA. DNA was re-suspended in 10µl of hybridization mix.

2.2.6.2.3. Pre-hybridization treatment of metaphase spreads

Slides were dehydrated in alcohol series, 70% - 90% -100% ethanol, 5 mins each at room temperature. Slides were air dried prior to treatment with 100µl RNAase (10 mg/ml RNAaseA stock diluted 1 µl in 100µl with 2x SSC) under a coverslip, and were then placed in a moist chamber at 37°C for 1 hour. This was followed by 5 min washes in 2x SSC at room temperature and incubation in proteinase K buffer at 37°C for 5 mins. Slides were then incubated at 37°C in proteinase K buffer with the addition of proteinase K to a final
concentration of 50 ng/ml for 7 mins. After fixing in paraformaldehyde buffer, the slides were dehydrated again in the alcohol series and air dried.

2.2.6.2.4. Hybridization
Probes with competitor DNA were incubated at 75°C for 4 mins to denature and were allowed to re-anneal at 37°C for 30 - 45 minutes prior to hybridization. Before hybridization metaphase chromosomes were denatured and dehydrated as in 2.2.3.4.2. After the slide was air dried, 10μl of re-annealed probe was applied to the slide under a coverslip and sealed with cow-gum. Overnight hybridization took place in a moist chamber at 37°C in an incubator.

2.2.6.2.5. Post-hybridization washes
Following hybridization, cow gum was removed and coverslips were soaked in 50% formamide / 2xSSC at 45°C until they came off. Washing was continued in 50% formamide / 2xSSC for a total of 15 mins including the initial soaking. Then, slides were washed in 2x SSC for 5 min at 45°C followed by a single wash in 0.1 x SSC for 5 min at 60°C. In preparation for signal detection slides were washed in SSCT followed by incubation in SSCM as in 2.2.3.3.3.

2.2.6.2.6. Single colour signal detection
The procedure was as in 2.2.3.3.4.
2.2.6.3. **Dual colour YAC FISH**

2.2.6.3.1. **Dual colour labelling of YAC DNA probes with first principle**

**Nick Translation**

The mixture consisted of 5μl of DNase I from bovine pancreas, grade II, (Boehringer Mannheim) (1:1000 dilution in water of 1mg/ml stock stored on ice and diluted just before use), 5μl of 0.1 M DTT (Sigma, 10g), 5μl of 10 x nick translation buffer, 4μl nucleotide mix (0.5 mM dA, dG, dC, 0.1mM dT), 2μl of 1mM biotin-16-dUTP (Boehringer) / 4 μl digoxigenin-11-dUTP (Boehringer) label, 3μl of DNA polymerase I (Promega, 7000U/ml, 500 units, kept in the freezer until required), to which was added 1 μg of probe DNA. This was made up to a final volume of 50μl with filtered distilled water and incubated for an hour at 16°C. Then 5μl of 0.5M EDTA (pH 8) stop buffer (Gibco) was added to stop the reaction.

2.2.6.3.2. **Simultaneous use of dual colour YACs and biotinylated alpha satellite DNA probes**

2.2.6.3.2.1. **Pre-hybridization preparation of probes**

200 ng each of two different colour, biotin and digoxigenin-labelled YAC DNA probes were prepared as in 2.2.6.2.2. Selected biotin-labelled alpha-satellite probes, commercially prepared, were supplied from Oncor.

2.2.6.3.2.2. **Pre-hybridization preparation of metaphase slides**

As in 2.2.6.2.3.

2.2.6.3.3. **Hybridization**

Biotin and digoxigenin-labelled YAC DNA probes and biotin-labelled alpha-satellite probe were denatured as in 2.2.6.2.4. The biotin-labelled alpha-satellite probe was put on ice while the biotin and digoxigenin-labelled YAC probes were pre-annealed with competitor as in 2.2.6.2.4.

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Then, the alpha-satellite probe was added to the YAC probes at a final concentration of 0.5 ng/ul and final volume of 10 μl. They were mixed well before being applied to a denatured slide (as in 2.2.3.4.2.).

2.2.6.3.4. Post hybridization washes

On the following day, coverslips were soaked in 50% formamide / 2 x SSC until they came off and washed for a total of 15 minutes including the initial soaking. This was followed by a 5 min wash in 2 x SSC at 45°C and 5 min in TN Tween at room temperature. After that they were incubated in TNB for 20 minutes.

2.2.6.3.5. Dual colour YAC probes and single colour alpha-satellite probe signal detection

The dual colour signal detection was carried out as follows:

1) 20 min incubation under cover at room temperature with avidin - fluorescein isothiocyanate (avidine-FITC) (1:200) and mouse anti-digoxigenin (1:1000) in TNB (100μl volume for each slide), followed by three 5 min washes in TN Tween at room temperature.

2) 20 min incubation in biotinylated - anti - avidin in SSCM (1:100) and TRITC rabbit anti-mouse (1:1000) in TNB (100μl volume for each slide) in the dark at room temperature, followed by three 5 min TNTween washes at room temperature.

3) 20 min incubation in the dark at room temperature with avidin - FITC (1:200) and TRITC goat anti-rabbit (1:1000) in TNB (as above) followed by one wash in TN Tween at room temperature and two washes in PBS for 5 min each.

Finally the chromosomes were counter-stained by the addition of 10 μl anti-fade containing only DAPI.
2.2.6.4. Image analysis

Chromosomes were visualised using a Nikon Fluorescence microscope. Single FISH signals were visualised through an FITC filter and double FISH signals were visualised through a dual band pass filter for simultaneous detection of FITC and TRITC. Images were analysed and stored using a Bio-Rad MRC 600 confocal laser microscope attachment with MRC 500/600 software (Figure 9).

Images were also captured, analysed and stored using a Zeiss Axioskop with a cooled CCD camera (Figure 10) and Smart Capture system (Digital Scientific- Cambridge).
2.2.7. Cosmids

2.2.7.1. Preparation of cosmid probes for FISH

2.2.7.1.1. Culture

A single colony was inoculated into 10 ml of cosmid broth, and incubated at 37°C with agitation overnight.

2.2.7.1.2. Extraction and preparation of human DNA from cosmids

The culture was centrifuged at high speed for 10 min. The supernatant was removed and incubated in 200μl of solution I (50mM Glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0) at room temperature for 10 min, followed by incubation in 400μl of solution II (0.2 M NaOH, 1% SDS) on ice for 5 min, followed by incubation in 300μl of solution III (3M Na acetate pH 5.5) on ice. This was mixed and centrifuged at 14,000 rpm for 15 min and then the supernatant was transferred to a fresh tube and 300μl of phenol was added. This was mixed and centrifuged for 3 min. DNA was precipitated with 100% ethanol from the supernatant then centrifuged at 14,000 rpm for 10 min and the DNA pellet was re-suspended in TE.
2.2.7.2. **Single colour cosmid FISH**

2.2.7.2.1. Single colour cosmid probes labelled using the BioNick kit

As in 2.2.6.2.1.

2.2.7.2.2. Pre-hybridization preparation of labelled probes

As in 2.2.6.2.2.

2.2.7.2.3. Pre-hybridization treatment of metaphase spreads

As in 2.2.6.2.3.

2.2.7.2.4. Hybridization

Cosmid probes were denatured and pre-annealed as in 2.2.6.2.4. Slides were denatured as in 2.2.3.4.2. and overnight hybridization was carried out as mentioned above.

2.2.7.2.5. Post-hybridization washes

As in 2.2.6.2.5.

2.2.7.2.6. Single colour cosmid signal detection

As in 2.2.3.3.4.

2.2.7.3. **Digoxigenin-labelling of cosmid DNA probes by first principle NT**

As in 2.2.6.3.1. by using only single labelling with digoxigenin-11-dUTP 4μl.
2.2.7.4. **Dual colour cosmid and paint FISH**

2.2.7.4.1. Simultaneous hybridization of digoxigenin labelled cosmid probe and biotinylated chromosome paint

2.2.7.4.1.1. Pre-hybridization preparation of probes

200 ng digoxigenin-labelled cosmid probe was prepared as in 2.2.6.2.2. The selected paint probe was supplied already biotin-labelled (Cambio).

2.2.7.4.1.2. Pre-hybridization preparation of metaphase slides

As 2.2.6.2.3.

2.2.7.4.2. Hybridization

Digoxigenin-labelled cosmid was denatured and pre-annealed as in 2.2.6.2.4. When this was ready, 10 μl of the biotin-labelled paint probe was added and hybridized as before.

2.2.7.4.3. Post-hybridization washes

As in 2.2.6.3.4.

2.2.7.4.4. Dual colour signal detection

As in 2.2.6.3.5.

2.2.7.5. Image analysis

As in 2.2.6.4
2.2.8. Genomic DNA extraction

2.2.8.1. Cell cultures

After removing the medium, monolayer cultures were first washed with Hanks and then with Versene solutions, then incubated in trypsin until all the cells came off from the culture bottle. Fetal calf serum was added to inactivate the trypsin before the suspension was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in 2 ml of 0.9% saline. This was centrifuged again at 1000 rpm and the supernatant was removed. The pellet was re-suspended and incubated in 2.5 ml of pH 8.5 Lysis buffer with proteinase K (100μg/ml). DNA was precipitated by isopropanol and dissolved in TE (pH 8.0) (10mM Tris Cl, 1mM EDTA & 100 ml distilled water).

2.2.8.2. Primary tumour

The tumour tissue was dissected into very small pieces (2mm³). To this was added 2 - 6 ml of reagent B (Nucleon II DNA extraction kit, Scotlab) and 500 - 1500 μl sodium perchlorate. The test tubes were then rotated on a daisywheel for 15 min at room temperature. This was followed by incubation in a waterbath at 65°C for 1 - 2 hours with manual inversion of test tubes occasionally. Afterwards, 2 - 5 mls of -20°C ice cold chloroform was added and tubes were gently shaken for 10 min at room temperature followed by centrifugation at 2000 rpm for a minute. Test tubes were again centrifuged at 2800 rpm for 3 min after 300 - 800 μl of Nucleon silica suspension was gently added down along the side wall of the test tubes. The clear DNA layer was transferred into a new test tube without disturbing the nucleon silica suspension layer. Finally DNA was precipitated using ice cold 100% ethanol.

2.2.8.3. Normal control blood samples

To 5 ml of fresh blood in 100 ml of EDTA was added 5 ml of TKM I (0.24 Tris, 0.15g EDTA, 0.15g KCl, 0.41g MgCl₂·6H₂O, 200ml distilled water). 125 μl of Noridet P 40 was used to lyse the cells. Solutions were mixed properly and centrifuged at 2200 rpm for 10 min and the supernatant was discarded. These steps were repeated until the red colour of
the pellet disappeared. Once the pellet was colourless, it was re-suspended in a few drops of TKM I, 0.8 ml of TKM II (200 ml TKM I with 4.68g NaCl) and 50μl 10% SDS. The solution was mixed by gentle pipetting before being incubated for 20 min at 55°C. 300 μl 6M NaCl was added before centrifuging at 12000 rpm for 5 min. DNA were precipitated from the supernatant using twice the volume of 100% ethanol at room temperature. The DNA was suspended finally in TE.
2.2.9. Comparative Genomic Hybridization

2.2.9.1. Labelling of genomic tumour DNA and normal control DNA by first principle NT

As in 2.2.6.3.1. except that DNAse I for the final working solution dilution was varied from 1:1000 - 1:2000 depending on the quality of hybridization required. Tumour genomic DNA was labelled with biotin and detected by FITC. Normal control genomic DNA was labelled with digoxigenin and detected by TRITC.

2.2.9.2. Preparation of labelled DNA for hybridization

Both biotin-labelled tumour genomic DNA and digoxigenin-labelled normal control DNA were prepared as in 2.2.6.2.2. Both DNAs were re-suspended in 7μl each of hybridization mix per probe.

2.2.9.3. Preparation of target normal human metaphase spreads

2.2.9.3.1. Blood culture

1 ml of heparinised whole blood was added to 17 ml Iscoves modified DMEM medium (Imperial), 2 ml GPS (glutamine, penicillin, streptomycin), 2 ml fetal calf serum in a 50 ml culture flask. 200μl of phytohaemaglutinin was added and the culture incubated for 72 hours at 37°C. After this time 200μl of 30 mg/ml thymidine (Sigma) was added to the culture and incubation was continued for another 18 hours at 37°C. 200μl of 0.227 mg/ml 2-deoxycytidine (Sigma) was then added and incubation continued for 3 hours 55 minutes at 37°C. 200 μl of 10μg/ml colcemid (Gibco) was added and incubated for 20 min at 37°C.

2.2.9.3.2. Harvest

As in 2.2.1.4.

2.2.9.3.3. Chromosome suspension and metaphase spreads

As in 2.2.1.5.
2.2.9.4. Pre-hybridization preparation of metaphase spreads

As in 2.2.6.2.3.

2.2.9.5. Hybridization

Both biotin-labelled tumour DNA and digoxigenin-labelled control normal DNA probes were denatured and pre-annealed as in 2.2.6.2.4. Slides were denatured as in 2.2.3.4.2. After the slide was air dried, 7μl each of reannealed probes (total of 14 μl) was applied to the slide under a coverslip and sealed with cow-gum. Hybridization was continued for four days in a moist chamber at 37°C.

2.2.9.6. Post-hybridization washes

As in 2.2.6.3.4.

2.2.9.7. Dual colour signal detection

Dual colour signal detection was as follows:

1) 20 min incubation under cover at room temperature with avidine - fluorescein isothiocyanate (avidine-FITC) (1:200) and mouse anti-digoxigenin (1:500) in TNB (100μl volume for each slide), followed by three 5 min washes in TN Tween at room temperature.

2) 20 min incubation in biotinylated - anti - avidine in SSCM (1:100) and TRITC rabbit anti-mouse (1:500) in TNB (100μl volume for each slide) under cover at room temperature, followed by three 5 min TNTween washes at room temperature.

3) 20 min incubation in the dark at room temperature with avidine - FITC (1:200) and TRITC goat anti-rabbit (1:500) in TNB (as above) followed by one wash in TN Tween at room temperature and washed twice in PBS for 5 min each.

Finally the chromosomes were counter stained by the addition of 10 μl anti - fade containing only DAPI.
2.2.9.8. Digital image analysis of CGH

Images were acquired using a multi-colour quantitative image processing system which is based on a regular fluorescence microscope, Zeiss Axiophot, equipped with a high resolution cooled (CCD) camera and filters (Figure 10). (see 2.2.6.4.)
Figure 10  Fluorescence microscopy with cooled coupled charged (CCD) camera

1) Zeiss Axiophot fluorescence microscope

2) Filters

3) CCD camera

4) CCD cooling box

5) Mercury lamp

6) Filter control

7) Macintosh system with Digital Scientific "Smart Capture" software
Chapter 3

Results
3. Results

3.1. Cell lines in culture

Cells grew as flat, adherent and polygonal cells as shown in Figure (11). Each line had a characteristic morphology, although XH1 and DE3 shared a very similar appearance.

Chromosome numbers

Modal numbers for the three squamous cell lines are shown in Figure 12 and chromosome counts for both early passage (Han et al 1991), and later passages of the adenosquamous line XH1 are shown in Figure 13. Since virtually all the cells from the adenocarcinoma JE6 had 52 chromosomes, no histogram is shown. With the exception of JE6, which was hyper-diploid, all modal numbers were in the hyper-triploid or hypo-tetraploid range.

Figure 11

a. SM7
b. EH2

c. DE3
d. JE6

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e. XH1
Figure 12  Histograms showing modal numbers of the three squamous cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>CHROMOSOMES/CELL</th>
<th>NO. CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM7</td>
<td>12</td>
<td>70717273747576777879808182838485868788899091929394959697</td>
</tr>
<tr>
<td>EH2</td>
<td>8</td>
<td>70717273747576777879808182838485868788899091929394959697</td>
</tr>
<tr>
<td>DE3</td>
<td>14-19</td>
<td>70717273747576777879808182838485868788899091929394959697</td>
</tr>
</tbody>
</table>
Figure 13  Histograms showing the number of chromosomes per cell in the adeno-squamous carcinoma cell line at different passage levels

![Histogram 1](chart1.png)

![Histogram 2](chart2.png)
3.2. SM7 (Squamous cervical carcinoma)

3.2.1. Karyotype by conventional G banding

Karyotyping of SM7 cells by G banding between passages 12 and 16, showed a modal chromosome number of 91, and a range of 88 - 96 (Histogram, Figure 12). Ten cells were fully karyotyped, and a representative cell is shown in Figure 14. The G band analysis for each chromosome was as follows:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of normal (N) and/or rearranged chromosomes and the number of cells in which they occurred.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>3N[10]</td>
</tr>
<tr>
<td>1</td>
<td>3N[3],[2N,del(1)(q25),der(1)add(1)(p31)[2], 2N, der(1)add(1)(p31)[1], 2N,del(1)(p21)[1], 3N,del(1)(p21)[1], 3N, der(1)add(1)(p31)[1], 2N,der(1)add(1)(p31),der(1)add(1)(p36)[1]</td>
</tr>
<tr>
<td>2</td>
<td>3N,der(2)add(2)(q11)[9], 3N[1]</td>
</tr>
<tr>
<td>3</td>
<td>3N,i(3q)[7], 2N,i(3q)[2], 3N, i(3q), del(3)(p22)[1]</td>
</tr>
<tr>
<td>4</td>
<td>3N[7], 2N[3]</td>
</tr>
<tr>
<td>5</td>
<td>5N[4], 4N[4], 3N[2]</td>
</tr>
<tr>
<td>6</td>
<td>3N,der(6)add(6)(q11)[10]</td>
</tr>
<tr>
<td>7</td>
<td>4N[5], 5N[4], 3N[1]</td>
</tr>
<tr>
<td>8</td>
<td>2N,i(8q)[6], 3N,i(8q)[4]</td>
</tr>
<tr>
<td>9</td>
<td>4N,i(9q)[4], 3N,i(9q)[2], 5N[2], 5N,i(9q)[1], 4N[1]</td>
</tr>
<tr>
<td>10</td>
<td>4N[9], 3N[1]</td>
</tr>
<tr>
<td>11</td>
<td>3N[5], 4N[4], 3N,der(11)add(11)(q22)[1]</td>
</tr>
</tbody>
</table>
12  $4N,\text{der}(12)t(8;12)(q11;p11)[8]$, $5N,\text{der}(12)[1]$, $3N, \text{der}(12)[1]$


14  $4N[8]$, $5N \ [2]$

15  $3N,\text{der}(15)\text{add}(15)(q22)[10]$


20  $5N[10]$


Eleven different breakpoints occurred in more than two cells. One marker which occurred in every cell looked as if one arm could have been 5p. Two other markers occurred once only in two different cells.
The main areas requiring further investigation with FISH were those rearrangements which occurred in all or a majority of cells:

1) The 2q rearrangement, der(2).
2) The 6q rearrangement, der(6)
3) Confirmation of the 8;12 translocation.
4) The addition to 13p, der(13).
5) The 15q rearrangement, der(15).
6) The identification of the metacentric marker, part of which could be 5p.

Any other hidden rearrangements present in most cells would be revealed by using all the chromosome specific paints separately, and then on the basis of the results, some paints would be selected to use in dual colour investigations.
Figure 14  G-banded Karyotype of SM7
(* chromosomes 1 from a different cell)
Figure 15
C-banded Karyotype of SM7

SM7

93 chromosomes
C-bands

1
der 2
i3q

4
5
?

der 6
7
i8q
i9q
10
11

der 12

der 13
14
der 15

16
17
18

19
20

21
22
X
3.2.2. Clarification of the SM7 karyotype by FISH chromosome painting

FISH chromosome painting was applied to metaphases of SM7 cells at passage 10 - 16 using twenty three chromosome specific paint probes (Chapter 2). Confocal images of FISH paints on metaphases from SM7 with chromosome rearrangements are shown in Figure 16.

The number of normal (N), and rearranged chromosomes was assessed for each paint in five or more cells. The results are shown in the following table:-

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Painted regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>3N, one arm of small metacentric slightly larger than chromosome 16, in most cells. (Fig.16f)</td>
</tr>
<tr>
<td>1</td>
<td>3N[5], 3N,der(1)add(1)(q21)[4], 3N,der(1)add(1)(p31)[3], 3N,der(1)add(1)(p36)[1] and multiple double minutes. (Fig.16 a &amp; b).</td>
</tr>
<tr>
<td>2</td>
<td>3N, der(2)add(2)(q11)[10] (Fig.16c)</td>
</tr>
<tr>
<td>3</td>
<td>3N, i(3q), short arm of a chromosome the size of a 6[10] (Fig.16d)</td>
</tr>
<tr>
<td>4</td>
<td>3N[7], 2N[2], 4N[1]</td>
</tr>
<tr>
<td>5</td>
<td>5N, one arm of a small metacentric marker slightly larger than a chromosome 16 in nearly all cells. (Fig.16e)</td>
</tr>
<tr>
<td>6</td>
<td>3N, der(6)add(6)(q11), distal 2/3 of the long arm of a small acrocentric the size of a small D, in most cells. (Fig.16g)</td>
</tr>
<tr>
<td>7</td>
<td>5N[4], 4N[4], 3N[2]</td>
</tr>
<tr>
<td>8</td>
<td>3N, i(8q), one arm of der (12)t(8;12) in most cells. (Fig.16i)</td>
</tr>
</tbody>
</table>
9 4N, i(9q) in most cells. Long arm of small metacentric instead of i(9q) in a few cells. (Fig.16n)

10 3 - 4 N, short arm of small submetacentric, the size of der(6) in most cells. (Fig.16h)

11 4N[6], 3N[2], 5N[1]

12 4N, one arm of der(12)t(8;12) in most cells. (Fig.16m)

13 2N, long arm of der(13)add(13)(p11)x2 in most cells. (Fig.16j)

14 4N[5]

15 2 - 3 N, proximal region of long arm of small acrocentric. (Fig.16i)

16 4N[8], 5N[1], 3N[1]

17 4N[10]

18 3N[8], 4N[2]

19 4N[7], 3N[2], 5N[1]

20 5N[9], 6N[1]

21 2N, short arm of small submetacentric the size of the der(13)x2 (Fig.16k)

22 4N[7], 3N[7]
Figure 16  Single colour FISH images from the SM7 cell line.

(a) Metaphase from SM7 with double minutes

(b) Metaphase from SM7 hybridized to paint 1, showing signal on sections of chromosome 1 and double minutes
(c) paint 2 shows: $3N$, der(2)t(2;3) (arrow)

(d) paint 3 shows: $3N$, der(2)t(2;3) (large arrow), i(3)(q10) (small arrow)
(e) paint 5 shows: 5N, der(X)t(X;5)q11;p12(arrow)

(f) paint X shows: 3N, der(X)t(X;5)q11;p12(arrow)

(g) paint 6 shows: 3N, der(6)t(6;10)q11;p11(large arrow) der(6)t(6;15)q21;q15(small arrow)

(h) paint 10 shows: 3N, der(6)t(6;10)q11;p11(arrow)
(i) paint 15 shows: 3N, der(15)t(6;15)(q21;q15)(arrow)

(j) paint 13 shows: 2N, der(21)t(13;21)(q12;p11)(arrow)

(k) paint 21 shows: 2N, der(21)t(13;21)(q12;p11)(arrow)
(l) paint 8 shows: 3N, der(8)t(8;12)(p10;q11)(arrow) i(8)(q10)(large arrow)

(m) paint 12 shows: 3N, der(8)t(8;12)(p10;q11)(arrow)

(n) paint 9 shows: 4N, i(9)(q10)(arrow)
Conclusions which could be drawn as a result of examining both G banding and FISH painting were as follows:-

1) The der(2) consisted of the short arm of 2 and part of the long arm of 3; der(2)t(2;3).

2) The der(6) consisted of the short arm of 6 and the short arm of 10; der(6)t(6;10)(q11;p11).

3) The 8;12 translocation was confirmed and could be written as der(8)t(8;12)(p10;q11).

4) The addition to 13p turned out to be a der(21)t(13;21)(q12;p11).

5) The der(15) had additional material from the long arm of 6 and could be written as der(15)t(6;15)(q21;q15).

6) The small metacentric marker was composed of 5p as suspected, translocated onto Xp; der(X)t(X;5)(q11;p12).
3.2.3. Clarification of breakpoints on chromosome 3 by using Cosmids

In order to clarify the breakpoints on chromosome 3, the following procedure was performed.

The centromeric alpha-satellite 2 probe was used to identify the marker, der(2)t(2;3). Both the digoxygenin labelled cosmid from 3p24 (red signals), and the biotin labelled cosmid from 3q27 (green signals), (idiogram illustrations, Figure 17a, and FISH image, (Figure 17b) were observed in the three normal copies of chromosome 3, but only the biotin labelled cosmid from 3q27 was seen on both arms of the isochromosome 3. On the der(2) marker, the green colour cosmid signals from 3q27 were present, hence the breakpoint was likely to be at 3q26.
Figure 17  Dual colour FISH image and idiograms

(a)  Idiogram illustrations of FISH image below

(b)  Dual colour FISH with co-hybridization of chromosome 2 alpha-satellite probe (large green signals), and chromosome 3 cosmids (red signals 3p24 & small green signals 3q27), to characterise the breakpoints as der(2)t(2;3)(q11;q26)
The full karyotype was therefore as follows:

88-96<4n>XXX, der(X)t(X;5)(q11;p12), -1, add(1)(p31)[5],

add(1)(q21)[4], der(2)t(2;3)(q11;q26), i(3)(q10), -4, +5,

der(6)t(6;10)(q11;p11), +7[8], +8[11], i(8)(q10), der(8)t(8;12)(p10;q11),

+9, i(9)(q10), -11[8], -13, -13, der(15)t(6;15)(q21;q15), -18, +20,

der(21)t(13;21)(q12;p11)x2[cp20] (Fig.18).

Figure (18) is a representative cell showing the revised karyotype of SM7 incorporating the FISH painting results. Breakpoints were determined from both G band photographs and confocal images.
Figure 18 Revised karyotype of SM7 after FISH painting
3.3. EH2 (Squamous cell carcinoma)

3.3.1. Karyotype by conventional G banding

Karyotyping of EH2 cells at passage 8 showed a modal chromosome number of 88 and a range of 87-91 (Figure 12). Ten cells were fully karyotyped and a representative cell is shown in Figure 19. The G band analysis for each chromosome was as follows:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of normal (N) and / or rearranged chromosomes and the number of cells in which they occurred.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>2N,der(X)add(X)(p22.1)x2[8], 2N,der(X)[2]</td>
</tr>
<tr>
<td>1</td>
<td>4N,der(1)add(1)(q21)[5], 3N,der(1)*[2], 3N,der(1)x2, der(1)add(1)(p11)[2], 4N[1]</td>
</tr>
<tr>
<td>2</td>
<td>3N[10]</td>
</tr>
<tr>
<td>3</td>
<td>3N[10]</td>
</tr>
<tr>
<td>4</td>
<td>1N,der(4)add(4)(q35)x2[8], 1N,der(4)[2]</td>
</tr>
<tr>
<td>5</td>
<td>4N[9], 3N[1]</td>
</tr>
<tr>
<td>6</td>
<td>2N,del(6)(p22),del(6)(q23)[6], 2N,del(6)(p)[1], 1N,del(6)(p),del(6)(q)x2[1], 2N,del(6)(q)x2[1], 1N, del(6)(q),+del(6)(p)x2[1]</td>
</tr>
<tr>
<td>7</td>
<td>5N[9], 4N[1]</td>
</tr>
<tr>
<td>8</td>
<td>3N,i(8q)[5], 2N,i(8q)[2], 3N[1], 4N[1], 2N, i(8q)x2[1]</td>
</tr>
<tr>
<td>9</td>
<td>4N,del(9)(p21)[7], 5N,del(9)(p)[1], 3N,del(9)(p)x2[1], 4N,del(9)(p)x2[1]</td>
</tr>
</tbody>
</table>

*breakpoints are not given where the same rearrangement is repeated.
Nine different breakpoints occurred in more than two cells. Two copies of a small acrocentric marker, M1, were found in every cell. Three other unidentified rearrangements were found in single cells only.
The main targets for FISH investigation were, therefore, as follows:-

1) To define the nature of the der 1 rearrangement.

2) To identify the addition to 4q.

3) To see whether the deletions to chromosome 6 were straightforward deletions, or rearrangements involving other chromosomes.

4) To do the same with the 9p deletion.

5) To identify the addition to 14p.

6) To identify the addition to Xp.

7) To identify the origin of the duplicated small acrocentric marker.

8) To find any other rearrangements not picked up with G banding.
Figure 19  G banded Karyotype of EH2
3.3.2. Clarification of the EH2 karyotype by FISH chromosome painting

FISH painting was applied to metaphases of EH2 cells at passage 12. Confocal images with rearrangements of EH2 are shown in the Figure 20. Figure 23 is the revised karyotype incorporating FISH painting results.

The number of normal (N) and rearranged chromosomes was assessed for each paint in four or more cells. The results are shown in the table below:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Painted regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>2N, derX x2 in most cells. The derX consisted entirely of X chromosome material so could be written as der(X)t(X;X)(p22;q13) (Fig.20j)</td>
</tr>
<tr>
<td>1</td>
<td>4N, der(1)add(1)(q21) in most cells. The paint stopped just below the 1q heterochromatic region. (Fig.20a)</td>
</tr>
<tr>
<td>2</td>
<td>3N[5]</td>
</tr>
<tr>
<td>3</td>
<td>4N, the distal 1/3 of the long arm of two B group sized chromosomes with an extended long arm in most cells. (Fig.20c)</td>
</tr>
<tr>
<td>4</td>
<td>1N, two copies of der(4) painted 2/3 of the way down the long arm the full length of the chromosome 4q in all cells. (Fig.20d)</td>
</tr>
<tr>
<td>5</td>
<td>4N[2], 3N[2]</td>
</tr>
<tr>
<td>6</td>
<td>2N,del(6)(p),der(6)add(6)(q21) with more than half the long arm painted in most cells. (Fig.20e)</td>
</tr>
<tr>
<td>7</td>
<td>5N[6], 4N[4]</td>
</tr>
<tr>
<td>8</td>
<td>3N,i(8q) in all cells</td>
</tr>
</tbody>
</table>

160
9 3N, del(9)(p21), del(9)(q21) in most cells. (Fig.20g). The paint on the normal 9's did not always cover the ends of the long arms. The painted region on the del 9q was exactly the size of 9p. The 9q heterochromatic region (DAPI bright) was apparent close to the centromere on the short arms of this chromosome.

10 4N[8], 3N[2]

11 3-4N in most cells and a region of paint on the short arm of a chromosome larger than a B group which looked like the der (1). (Fig.20b)

12 4N[4], 3N[2], 8N[1]

13 4N[4], 5N[1]

14 Two small acrocentrics the size of an F group, and two submetacentrics slightly bigger than a 9, painted entirely. (Fig.20i) These can, therefore, be written as del(14)(q23) and der(14)t(14;14)(p11;q23).

15 4N[3], 8N[1], 12N[1]

16 3N[2], 4N[2], 7N[1]

17 3-4 N mostly, a region of paint about 1/2 the length of 17q was found at the distal end of a C group chromosome der(?)t(?,17) and often present as two copies. (Fig.20f)

18 6N[3], 4N[1], 3N[1]

19 4N[2]

20 6N[4], 5N[1]

161
<table>
<thead>
<tr>
<th>Page</th>
<th>Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>7N[2], 4N[2], 2N[1]</td>
</tr>
<tr>
<td>22</td>
<td>3N[4], 2N[3], 6N[1]</td>
</tr>
</tbody>
</table>
Figure 20  Single colour FISH images from the EH2 cell line

(a) paint 1 shows: 4N, der(1)t(1;11) (arrow)

(b) paint 11 shows: 4N, der(1)t(1;11) (arrow)

(c) paint 3 shows: 4N, der(4)t(3;4) x2 (arrows)

(d) paint 4 shows: 1N, der(4)t(3;4) x2 (arrows)
(e) paint 6 shows: 2N, 
der(6)t(6;17)x2(arrows)

(f) paint 17 shows: 4N, 
der(6)t(6;17)x2(arrows)

(g) paint 9 shows: 2N, 
del(9)(p21)(large arrow) 
del(9)(q21)(small arrow)

(h) paint 8 shows: 3N, i(8q)(arrow)
(i) paint 14 shows: der(14)t(14;14)(p11;q23)x2 (large arrows)
    del(14)(q23)x2 (small arrows)

(j) paint X shows: 2N, der(X)t(X;X)(p22;q13)x2 (arrows)
As a result of the FISH painting and G banding, the following conclusions could be drawn:

1) The der 1 rearrangement showed a breakpoint at 1q21 with a region of chromosome 11 attached, probably 11q with a breakpoint in q13 or q14, so that it could be written as der(1)t(1;11)(Figure 20 a & b).

2) The additional material on 4q clearly came from the long arm of chromosome 3; der(4)t(3;4)(Figure 20 a & b).

3) The del(6)(p) was painted entirely, and did not appear to involve any other chromosome. The del(6)(q) was not painted throughout the long arm, but only down to q21. The remainder probably came from chromosome 17.

4) The del(9)(p) did not involve any other chromosome. A del(9)(q21) was found by FISH which had previously been designated by G banding as an extra 17.

5) The addition to chromosome 14p was found to be also material from chromosome 14.

6) Likewise the addition to the X was also material from an X chromosome.

7) The small duplicated acrocentric chromosome was derived from chromosome 14.

8) No other rearrangements which had not been picked up with G banding were found.
3.3.3. Dual colour FISH

In order to clarify the der(1), a dual colour FISH experiment was carried out, using concurrently, FITC directly labelled paint 1, detected as green signals, and biotin labelled paint 11, detected by texas red, visualised as red signals (Figure 21a). Four copies of normal chromosome 1 (green signals) and four copies of the normal chromosome 11 (red signals) with der(1) (green & red, dual coloured chromosome) were seen. Therefore the der(1) was confirmed as der(1)t(1;11)(q21;q13)(Figure 21a).

The same procedure was repeated by using FITC directly labelled paint 1 and biotin labelled paint 8 simultaneously. Four copies of the normal chromosome 1 and the der(1) with an unpainted lower end were detected as a green colour. Three copies of normal chromosome 8, and the long arms of the isochromosome 8 were detected by texas red and visualised as red signals. In this experiment, the red signal (chromosome 8 paint) was not seen on der(1). This doubly confirmed the der(1) as above, and excluded the possibility of it being t(1;8) (Figure 21b).

Two separate single colour FISH paints, 6 and 17 raised the possibility of der(6) being a straightforward rearrangement between chromosome 6 and 17. Dual colour FISH was set up by using FITC directly labelled paint 6, detected as green signals and biotin labelled paint 17, detected by texas red seen as red signals (Figure 21c). There were two copies of chromosome 6 (green signals), four copies of chromosome 17 (red signals) and a dual-coloured chromosome which could be concluded was der(6)t(6;17)(q21;q21).
Figure 21  Dual colour FISH images

(a) FITC labelled paint 1 (green signals) and biotin labelled paint 11 detected by texas red (red signals) to characterise der(1)t(1;11)(q21;q13)(arrow)

(b) FITC directly labelled paint 1 (green signals) and biotin labelled paint 8, detected by texas red (red signals)
Dual colour FISH using FITC directly labelled paint 6 (green signals) and biotin labelled paint 17, detected by texas red (red signals)
3.3.4. Clarification of breakpoints on chromosome 3 by using YACs

In EH2, signals from the 3q21 YAC were only observed on the three normal copies of chromosome 3 and not on the der(4)t(3;4) marker (Figure 22a). Therefore, the breakpoint must be lower than 3q21. Signals from the 3q24 YAC were seen on the marker as well as on the normal chromosome 3 (Figure 22b), so the breakpoint was assigned to 3q22.
Figure 22  Single colour FISH with YAC

(a) YAC 3q21 signals were observed on 3 copies of the normal chromosome 3 (small arrows), but not on the der(4) (large arrows).

(b) YAC 3q23-24 signals were observed on der(4)(large arrows) and characterise the breakpoints as der(4)t(3;4)(q22;q35) (small arrows show 3 copies of normal chromosome 3)
As a result of all the investigations, the full karyotype was therefore as follows;

88<4n>XX, der(X)t(X;X)(p22;q13)x2, +der(1)t(1;11)(q21;q13), -2,
-3, -4, der(4)t(3;4)(q22;q35)x2, der(6)t(6;17)(q21;q21)x2,
del(6)(p22), +7, i(8)(q10), +9, del(9)(p21), del(9)(q21), -12,
der(14)t(14;14)(p11;q23)x2, del(14)(q23)x2, -16, -17, -18, +20, +21, -22 (Fig.23).
Figure 23 Revised karyotype of EH2 after FISH painting
3.4. **DE3 (Squamous cell carcinoma)**

3.4.1. **Karyotype by conventional G banding**

Karyotyping of DE3 cells between passages 14 and 19 showed a modal chromosome number of 77 and a range of 74-80 (Figure 12). Ten cells were fully karyotyped and a representative cell is shown in Figure 24. The G band analysis for each chromosome was as follows:-

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of normal (N) and/or rearranged chromosomes and the number of cells in which they occurred.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>2N, del(X)(p11)[7], 1N, del(X)[1], 2N[2]</td>
</tr>
<tr>
<td>1</td>
<td>i(1q)x2, i(1p)x2[7], i(1q), i(1q)[1], i(1p)x2[3]</td>
</tr>
<tr>
<td>2</td>
<td>3N[10]</td>
</tr>
<tr>
<td>3</td>
<td>3N, i(3q)[10]</td>
</tr>
<tr>
<td>4</td>
<td>2N[10]</td>
</tr>
<tr>
<td>5</td>
<td>4N[9], 3N[1]</td>
</tr>
<tr>
<td>6</td>
<td>3N[9], 2N[1]</td>
</tr>
<tr>
<td>7</td>
<td>5N[6], 4N[3], 3N[1]</td>
</tr>
<tr>
<td>8</td>
<td>2N, i(8q)[10]</td>
</tr>
<tr>
<td>9</td>
<td>3N, der(9)add(9)(p22)x3[7], 2N, der(9)x3[1], 3N, der(9)[1], 3N, der(9)x2[1]</td>
</tr>
<tr>
<td>10</td>
<td>3N[10]</td>
</tr>
<tr>
<td>11</td>
<td>2N[10]</td>
</tr>
<tr>
<td></td>
<td>Chromosome Configuration</td>
</tr>
<tr>
<td>-----</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>12</td>
<td>2N, del(12)(p11)[5], 3N[4], 2N[1]</td>
</tr>
<tr>
<td>13</td>
<td>3N[8], 2N[2]</td>
</tr>
<tr>
<td>14</td>
<td>der(14)add(14)(q32)x3[8], der(14)x2[2]</td>
</tr>
<tr>
<td>15</td>
<td>3N, der(15)add(15)(p11)[6], 2N, der(15)[4]</td>
</tr>
<tr>
<td>16</td>
<td>3N[9], 2N,i(16q)[1]</td>
</tr>
<tr>
<td>17</td>
<td>3N[9], 4N[1]</td>
</tr>
<tr>
<td>18</td>
<td>3N[6], 4N[2], 2N[2]</td>
</tr>
<tr>
<td>19</td>
<td>1N[10]</td>
</tr>
<tr>
<td>20</td>
<td>4N[5], 3N[4], 2N[1]</td>
</tr>
<tr>
<td>21</td>
<td>4N[6], 3N[3], 2N[1]</td>
</tr>
<tr>
<td>22</td>
<td>5N[8], 6N[1], 4N[1]</td>
</tr>
</tbody>
</table>

Eight different breakpoints occurred in more than two cells. One unidentified marker the size of a C group, and a small pale fragment were seen in every cell. A small metacentric, the size of an E group was also found in three cells.

The main targets for the FISH painting would, therefore, be to identify:-

1) The addition to 9p.
2) The addition to 14q.
3) The addition to 15p.
4) The location of apparently absent chromosome 19 material.
5) The explanation for so many copies of chromosome 22.
6) The identification of the C group marker.
7) The identification of the small pale fragment.
8) Identification of any hidden rearrangements.
3.4.2. Clarification of the DE3 karyotype by FISH chromosome painting

FISH chromosome painting was applied to metaphases at passage 14 - 25. Confocal images with rearranged chromosomes are shown in Figure 25, and the revised karyotype incorporating the FISH results is shown in Figure 27.

The number of normal (N) and rearranged chromosomes assessed for each paint is shown in the Table below:

<table>
<thead>
<tr>
<th>Chromosomes</th>
<th>Painted regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>2N, delX entirely painted (Fig.25k)</td>
</tr>
<tr>
<td>1</td>
<td>i(1p)x2, i(1q), del i(1q), del(1)(p12), a section of the distal part of the long arm of a C-group-sized-chromosome in most cells. (Fig.25 a &amp; b)</td>
</tr>
<tr>
<td>2</td>
<td>3N [10]</td>
</tr>
<tr>
<td>3</td>
<td>3N, i(3q)[10] (Fig.25d)</td>
</tr>
<tr>
<td>4</td>
<td>2N, and the short arm of a D group sized chromosome[6], 2N[4] (Fig.25e)</td>
</tr>
<tr>
<td>5</td>
<td>4N[10]</td>
</tr>
<tr>
<td>6</td>
<td>3N[5], 2N, top half of a B group sized chromosome [5]</td>
</tr>
<tr>
<td>7</td>
<td>5N[8], 4N[2]</td>
</tr>
<tr>
<td>8</td>
<td>2N, i(8q)[5], 2N, i(8q)x2[5] (Fig25g)</td>
</tr>
<tr>
<td>9</td>
<td>3N, three copies of the der(9)(p) with the short arm entirely painted (Fig.25h)</td>
</tr>
<tr>
<td>10</td>
<td>3N[10]</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>2N[10]</td>
</tr>
<tr>
<td>12</td>
<td>3N[10]</td>
</tr>
<tr>
<td>13</td>
<td>3N[10]</td>
</tr>
<tr>
<td>14</td>
<td>Three large acrocentrics with the distal end of the long arm not painted [10] (Fig.25i)</td>
</tr>
<tr>
<td>15</td>
<td>2N, der(15) with p arm unpainted [5], 3N, der (15) as above [5] (Fig. 25f)</td>
</tr>
<tr>
<td>16</td>
<td>3N[10]</td>
</tr>
<tr>
<td>17</td>
<td>3N[10]</td>
</tr>
<tr>
<td>18</td>
<td>3N[10]</td>
</tr>
<tr>
<td>19</td>
<td>1N, small acrocentric x 2, distal part of long arm of D group chromosome x 3, in most cells. (Fig.25j)</td>
</tr>
<tr>
<td>20</td>
<td>3N, short arm, centromere and part of long arm of medium sized C group chromosome in most cells. (Fig.25c)</td>
</tr>
<tr>
<td>21</td>
<td>3N[10]</td>
</tr>
<tr>
<td>22</td>
<td>3N[10]</td>
</tr>
</tbody>
</table>
Figure 25  Single colour FISH images from the DE3 cell line

(a) paint 1-shows:

\[ \text{del}(1)(p12)^a \]
\[ i(1q)^b \]
\[ \text{del} i(1q)^c \]
\[ i(1p)x^2d \]
\[ \text{der}(20)t(1;20)(q21;p11)^e \]

---

b. Idiogram  illustrations of cell above

\[ \text{del} 1(p12) \]
\[ i(1q) \]
\[ \text{del} i(1q) \]
\[ i(1p)x^2 \]
\[ \text{der}(20) \]
(c) paint 20 shows: 3N, der(20)t(1;20)(q21;p11)(arrow)

(d) paint 3 shows: 3N, i(3)(q10)(arrows)
(e) paint 4 shows: 2N, der(15)t(4;15)(q33;p11)(arrow)

(f) paint 15 shows: 2N, der(15)t(4;15)(q33;p11)(arrow)

(g) paint 8 shows: 2N, i(8)(q10)x2 (arrows)

(h) paint 9 shows: 3N, der(9)t(9;9)(p22;q33)x3 (arrows)
(i) paint 14 shows: der(14)t(14;19)(q32;p12) (arrows)

(j) paint 19 shows: 1N, der(14)t(14;19)(q32;p12)x3 (three large arrows), del(19)(p12)x2 (two small arrows)

(k) paint X shows: 2N, del(X)(p11)(arrow)
As a result of both G band and FISH paint analysis, the following conclusions could be drawn:-

1) The addition to 9p consisted entirely of chromosome 9 material.

2) The pale G band region at the distal end of the long arm of chromosome 14 came from chromosome 19 (Figure 25 i & j).

3) The addition to 15p consisted of chromosome 4 material (Figure 25 e & f).

4 & 5) The chromosome 19 paint showed that two chromosomes, which on G banding looked like 22's, were in fact deleted 19's (Figure 25j).

6) The C group marker turned out to be a der 20 with chromosome 1 material comprising most of the long arm, der(20)t(1;20)(q21;p11) (Figure 25c).

7) The small pale centric fragment was a deleted 19 (Figure 25j).

8) No hidden rearrangements, apart from the 19 deletions, were picked up, which had not been suspected from the G banding.

All the other rearrangements seen with G banding, were confirmed with FISH. Several additional changes picked up with FISH such as the del i(1q), the del(1)(p12), the rearrangements of chromosome 6, and duplication of the i(8q) tended to occur at later passages.
3.4.3. **Analysis on chromosome 9 using single colour FISH with a cosmid probe**

The cosmid cloned from chromosome 9q34 (chapter 2.1.4) (Table 4) was used to reveal the rearrangement on der (9)(p), fully painted by the chromosome 9 paint.

The telomeric end of the short arm of this rearranged chromosome was from 9q34, and could be written as der(9)t(9;9)(p22;q33) (Fig. 26)

**Figure 26  Single colour FISH with a cosmid probe**

Cosmid from 9q34 to define the breakpoints as der(9)t(9;9)(p22;q33)

der (9) x3 (large arrows) ;  3N (small arrows)
As a result of all these investigations, the final karyotype of DE3 was as follows;

- X,
77<4n>XX, del(X)(p11), i(1)(p10)x2, i(1)(q10)x2, -2, i(3)(q10), -4, -4, -6, -7, -8, i(8)(q10), +9, der(9)t(9;9)(p22;q33)x3, -10, -11, -11, -12, -13, -14, der(14)t(14;19)(q32;p12)x3, der(15)t(4;15)(q33;p11), -16, -17, -18, -19,del(19)(p12)x2, +der(20)t(1;20)(q21;p11), -22 (Fig. 27).
Figure 27. Revised karyotype of DE3 after FISH painting
3.5. JE6 (Adenocarcinoma)

3.5.1. Karyotype by conventional G banding

Chromosome preparations for G and C banding at passages 8 and 9 showed a modal number of 52 (22 out of 30 cells) and a range between 50 and 54. A representative cell is shown in Figure 28. G band analysis for each chromosome was as follows:-

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of normal (N) and/or rearranged chromosomes and the number of cells in which they occurred.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>3N[9], 2N[1]</td>
</tr>
<tr>
<td>1</td>
<td>2N, i(1q)[10]</td>
</tr>
<tr>
<td>2</td>
<td>1N, der(2)t(2;7)(q31;q36)x2[8], 1N, der(2)[2]</td>
</tr>
<tr>
<td>3</td>
<td>1N, der(3)t(3;5)(p24;q31)[10]</td>
</tr>
<tr>
<td>4</td>
<td>2N[10]</td>
</tr>
<tr>
<td>5</td>
<td>1N, der(5)t(3;5)(p24;q31)[10]</td>
</tr>
<tr>
<td>6</td>
<td>2N[9], 3N[1]</td>
</tr>
<tr>
<td>7</td>
<td>1N, der(7)t(2;7)(q31;q36)[10]</td>
</tr>
<tr>
<td>8</td>
<td>2N[10]</td>
</tr>
<tr>
<td>9</td>
<td>2N, del(9)(q13)[10]</td>
</tr>
<tr>
<td>10</td>
<td>3N[9], 2N[1]</td>
</tr>
<tr>
<td>11-22</td>
<td>2N[10]</td>
</tr>
</tbody>
</table>
Six different breakpoints occurred in more than two cells. A G-negative small fragment was found in nearly every cell. Other markers were found in single cells only.

The main reasons for doing FISH on this culture were:-

1) To confirm the origin of the two translocations (2;7) and (3;5).
2) To see whether del(9) was a simple deletion or part of a rearrangement.
3) To find the origin of the small G-negative fragment.
Figure 29  C banded Karyotype of JE6
3.5.2. Clarification of the JE6 karyotype by FISH chromosome painting

FISH painting was applied to metaphases of JE6 cells at passage 9 - 14 and confocal images with chromosome rearrangements are shown in Figure 30.

FISH painting results are shown below:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Painted region</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>3N</td>
</tr>
<tr>
<td>1</td>
<td>2N, i(1q) (Fig. 30a)</td>
</tr>
<tr>
<td>2</td>
<td>1N, del(2)(q31)x2, lower half of the long arm of the der(7) (Fig.30b)</td>
</tr>
<tr>
<td>3</td>
<td>1N, regions on two products of the translocation; der(3)t(3;5)(p24;q31) (Fig.30c)</td>
</tr>
<tr>
<td>4</td>
<td>2N</td>
</tr>
<tr>
<td>5</td>
<td>1N, regions on two products of the translocation; der(3)t(3;5)(p24;q31) (Fig.30d)</td>
</tr>
<tr>
<td>6</td>
<td>2N</td>
</tr>
<tr>
<td>7</td>
<td>1N, der(7)t(2;7)(q31;q36)</td>
</tr>
<tr>
<td>8</td>
<td>2N</td>
</tr>
<tr>
<td>9</td>
<td>2N, del(9)(q13)(Fig30e)</td>
</tr>
<tr>
<td>10</td>
<td>3N</td>
</tr>
<tr>
<td>Chromosome Set</td>
<td>Description</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>11-19</td>
<td>2N</td>
</tr>
<tr>
<td>20</td>
<td>2N, small chromosome the size of 20p (Fig30f)</td>
</tr>
<tr>
<td>21-22</td>
<td>2N</td>
</tr>
</tbody>
</table>
Figure 30 Single colour FISH images from the JE6 cell line

(a) paint 1 shows: 2N (small arrows), i(1)(q10) (large arrow)

(b) paint 2 shows: 1N, del(2)(q31)x2 (small arrows),
    der(7)t(2;7)(q31;q36) (large arrow)
(c) paint 3 shows: 1N, t(3;5)(large arrows)

(d) paint 5 shows: 1N, t(3;5) (large arrows)

(e) paint 9 shows: 2N, del(9)(q13)(arrow)

(f) paint 20 shows: 2N, del(20)(q10)(arrow)
FISH painting showed that:

1) The translocations (2;7) and (3;5) were both confirmed and the breakpoints refined.

2) The del(9) was a simple deletion and was not part of a rearrangement.

3) The small G-negative fragment was composed of chromosome 20 material, probably the short arm.
3.5.3. Clarification of breakpoints on chromosome 3 by using YAC probe

3p25 YAC signals were seen on the normal chromosome 3 and on the der(5) translocation product, but were absent from the der(3), showing that the breakpoint was below 3p25, so that the translocation could be written t(3;5)(p24;q31)(Figure 31)

Figure 31  Single colour FISH with YAC probe

YAC from 3p25 to characterise the breakpoint on der(3)
IN (small arrow) ; t(3;5) (large arrow)
The final karyotype of JE6 was as follows:

- $52 < 2n > XX$, +X, +i(1)(q10), +del(2)(q31), +t(3;5)(p24;q31),
- der(7)t(2;7)(q31;q36), +del(9)(q13), +10, +del(20)(q10) (Fig. 32).
Figure 32  Revised karyotype of JE6 after FISH painting
3.6. XH1 (Adeno-squamous carcinoma)

3.6.1. Karyotype by conventional G banding

Cells from XH1 were the first of the cervical cancer cell lines to be karyotyped at the Galton Laboratory. (Han et al 1991). At passage 6, they had a modal number of 78 and a range of 75 - 95(Figure 13). Ten cells were fully karyotyped at the time, and a representative cell is shown in Figure 33. The G band analysis for each chromosome was as follows:-

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Normal number (N) and / or rearranged chromosomes, and the number of cells</th>
<th>in which they occurred</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>4N[8], 3N[1]</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2N, der(1)add(1)(p36)[2], 3N, der(1)add(1)(p)[2],</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2N, der(1)add(1)(p), der(1)add(1)(q11)[1]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4N, der(1)add(1)(p)[1], 2N, der(1)add(1)(q)[1], 4N[1],</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3N, der(1)add(1)(q21)[1], 3N, del(1)(p11)[1]</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4N[6], 3N[4]</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3N, der(3)add(3)(p13)[7], 4N[2], 3N[2]</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3N[5], 2N[5]</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3N[6], 4N[3], 2N[1]</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3N[8], 4N[2]</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4N[10]</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4N[9], 3N[1]</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4N[9], 3N, der(9)add(9)(p24)[1]</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3N, der(10)add(10)(p11) ie 10p+[5], 3N[2], 4N[2]</td>
<td></td>
</tr>
</tbody>
</table>

199
2N, der(10)add(10)(p11), der(10)add(10)(p11)*ie 10p- [2]

11 der(11)add(11)(p11)x2, der(11)add(11)(q23)x2[5]
der(11)add(11)(p)x2, der(11)add(11)(p11)*, der(11)add(11)(q)[1]
der(11)add(11)(p)x3, der(11)add(11)(q)x2[1]
der(11)add(11)(p), der(11)add(11)(p11)*, der(11)add(11)(q)[1]
der(11)add(11)(q)x2, der(11)add(11)(p)[1]

12 2N,del(12)(p12)x2[7], 2N,del(12)(p12)[3]

13 2N,der(13)add(13)(p11)[5], 1N,der(13)add(13)(p)[4],
3N,der(13)add(13)(p)[1]

14 der(14)add(14)(p11)x2[5], 2N, der(14)add(14)(p)x2[3]
1N,der(14)add(14)(p)x2[2]

15 3N[4], 1N[3], 2N[3]

16 4N[4], 3N[3], 2N[2], 3N,i(16q)[1]

17 3N [7], 4N [3]

18 2N[10]

19 2N[7], 3N[3]

20 4N [9], 3N [1]

21 2N[7], 1N[3]

22 3N[6], 1N[2], 2N[2]

*different from previous der

Nine different breakpoints occurring in more than two cells were found.
Markers which could not be identified are shown in the following table:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Description</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Large submetacentric the size of a 2</td>
<td>3/10</td>
</tr>
<tr>
<td>M2</td>
<td>Submetacentric the size of an 8</td>
<td>5/10</td>
</tr>
<tr>
<td>M3</td>
<td>Metacentric, the size of a 16, with dark bands on both arms, sometimes present as two copies</td>
<td>7/10</td>
</tr>
<tr>
<td>M4</td>
<td>Metacentric, the size of an F group, nearly always as two copies, one arm light, the other dark</td>
<td>8/10</td>
</tr>
<tr>
<td>M5</td>
<td>Very small metacentric the size of a G group, nearly always present as two copies</td>
<td>every cell</td>
</tr>
</tbody>
</table>

Three other unidentified marker chromosomes which were not found in later passages were:

1) A large acrocentric the length of a B group chromosome (Han et al 1991) which could have been a \( ^{3/18} \) translocation, der(18)t(3;18)(q11;q23) and occurred in \( ^{4/10} \) cells.

2) A large submetacentric, the size of a 2 which could have been a 15p+, (Han et al 1991), and was found in \( ^{2/10} \) cells.

3) A small acrocentric slightly larger than a G.

(\( M1 \) and \( M2 \) are not in Figure 33)
Figure 33  G banded karyotype of XHI (passage 6)

80 chromosomes

der 1p 2 der 3 4 5

6 7 8 9 der 10p+ der11p der11q del 12

der 13 der 14 15 16 17 18

19 20 M M3 M4 M5

11 21 22 X

M and M did not occur in later passages, M1 and M2 did not occur in this cell.
3.6.2. Cells from XH1 were re-examined in this laboratory between passages 35 and 39. At this stage the modal chromosome number had shifted to 83 with a range between 74 and 86 (Fig. 13). Ten cells were fully karyotyped, five at passage 35 and five at passage 39. A representative cell is shown in Figure 34. The G band analysis for each chromosome was as follows:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Number of normal(N) and / or rearranged chromosomes, and the number of cells in which they occurred.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>4N[9], 5N[1]</td>
</tr>
<tr>
<td>1</td>
<td>2N, der(1)add(1)(p36), der(1)add(1)(q11)[3]</td>
</tr>
<tr>
<td></td>
<td>1N, der(1)(p)*x2, der(1)(q)[2], 2N, der(1)(p), del(1)(p11)[1],</td>
</tr>
<tr>
<td></td>
<td>2N, der(1)(p)[1], 1N, der(1)(p), i(1p)[1],</td>
</tr>
<tr>
<td></td>
<td>1N, der(1)(p)x2[1], 1N, der(1)(p)x2, i(1p)[1]</td>
</tr>
<tr>
<td>2</td>
<td>3N[6], 4N[4]</td>
</tr>
<tr>
<td>3</td>
<td>3N, der(3)add(3)(p13)[9], 2N, der(3)(p)[1]</td>
</tr>
<tr>
<td>4</td>
<td>3N[5], 2N[5]</td>
</tr>
<tr>
<td>5</td>
<td>4N[7], 5N[2], 3N[1]</td>
</tr>
<tr>
<td>6</td>
<td>3N[8], 4N[2]</td>
</tr>
<tr>
<td>7</td>
<td>2N[6], 3N[3], 4N[1]</td>
</tr>
<tr>
<td>8</td>
<td>4N[8], 5N[2]</td>
</tr>
<tr>
<td>9</td>
<td>4N[8], 3N[2]</td>
</tr>
<tr>
<td>10</td>
<td>3N, der(10)add(10)(p11)ie 10p*[4] only at passage 39, 4N[3], 3N[3]</td>
</tr>
</tbody>
</table>

*where the breakpoints of a der are not specified they are as described initially.
Ten different breakpoints occurred in more than two cells. Two copies of M5 and one of M2 were found in every cell. M4 was found in eight out of ten cells and M1 in seven. M3 and M6 were each found in six cells, and a larger marker of unknown origin was found in one cell only.
XH1 comparisons between passage 6 and passage 35-39

Similarities

1. Both showed a variety of chromosome 1 rearrangements, some only occurring in a few cells, but the same rearrangement, der(1)add(1)(p36), was found in a majority of the cells at both early and late passages.

2. The same chromosome 3 rearrangement, der(3)add(3)p(q3), appeared in nearly all the cells.

3. Two copies of two different chromosome 11 rearrangements, der(11)add(11)(p11) and der(11)add(11)(q23), were found in most cells throughout the culture period.

4. There were two copies of a deleted 12(p12) in most cells at both passage levels.

5. The same der(13) and der(14) occurred in every cell at both passages.

6. Markers M1, M2, M3, M4 and M5 were found throughout.

7. Similar numbers of chromosomes 2, 4, 6, 8, 9, 15, 20, 21, 22 and X were found.
Differences

1. A der(10), 10p+, was apparent at passage 6, but was not detectable at passage 35. It was replaced by a 10p- chromosome at passage 39.

2. The del(16)(p11) was also only found at passage 39.

3. Two larger markers, the submetacentric which could have been a 15p+ and the acrocentric, possibly a 3/18 translocation, were found in some of the cells. They were only present at passage 6. Similarly, a small acrocentric marker slightly larger than a G chromosome, which never occurred in more than four out of ten cells, and was not given a number, had disappeared by the 35th passage. All these rearrangements must have either been selected out, or changed by subsequent culturing.

4. M6, a small submetacentric, the size of an F group, only occurred at later passages.

5. There was a general tendency for the number of chromosomes per cell to increase with time in culture. Chromosomes 5, 14 and 17 tended to build up their numbers with time, although a reduced number of 7's was apparent with increasing passage level.

The main targets for FISH investigation were to identify the six markers M1 - M6, and to find out the origin of the additions to chromosomes 1, 3, 10, 11, 13 and 14.
3.6.3. Clarification of the XH1 karyotype by FISH chromosome painting

FISH painting was applied to metaphases of XH1 cells at passage 35 - 39. The number of normal (N) and rearranged chromosomes was assessed for each paint in five or more cells. Confocal images with chromosome rearrangements are shown in Figure 35.

The results are as follows:

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>painted regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>5N[9], 4N[6]</td>
</tr>
<tr>
<td>1</td>
<td>2N, der(1)add(1)(p36), der(1)add(1)(q11), small region on the end of the short arm of a chromosome the size of an X. [10] (Fig.35a).</td>
</tr>
<tr>
<td>2</td>
<td>3N, distal 2/3 of long arm of a chromosome the size of a 2 M1, (Fig.35g) in most cells.</td>
</tr>
<tr>
<td>3</td>
<td>3N, del(3)add(3)(p14) in most cells (Fig.35c)</td>
</tr>
<tr>
<td>4</td>
<td>2N, a chromosome the size of a 16 entirely painted, probably del(4)(q21), M3, (Fig.35i) in most cells.</td>
</tr>
<tr>
<td>5</td>
<td>5N, a chromosome the size of a 16, entirely painted, probably del(5)(q13), M3, (Fig.35j)[5], 4N,del(5)[3], 3N,del(5)[2]</td>
</tr>
<tr>
<td>6</td>
<td>3N, distal region of a large chromosome, der(1)add(1)(p36), short arm of C group(Fig.35e), long arm of which looks like a 10q on DAPI, in most cells.</td>
</tr>
<tr>
<td>7</td>
<td>2-3N, short arm and proximal part of long arm of chromosome the size of a 2, ie der(7)add(7)(p15), M1, (Fig.35h) in most cells.</td>
</tr>
<tr>
<td>8</td>
<td>4N[6], 3N,long arm of chromosome 8-sized [4].</td>
</tr>
</tbody>
</table>
9 4N, [6], 3N[4]

10 3N, der(10)add(10)(p11)[7], 2N, der(10)[2], 1N, der(10)x2[1] (Fig35f)

11 No normals, der(11)add(11)(q23)x2 entirely painted, long arm of chromosome smaller than an 11 x 2 in all cells. (Fig.35k)

12 2N, del(12)(p11), long arm of chromosome like a large D [5](Fig35m), 3N, del(12)(p11)[5]

13 1N, der(13)add(13)(p11) entirely painted, short arm of a chromosome the size of an X (M2), long arm of a chromosome the size of a 16 (M4), (Fig.35p) in all cells.

14 2N, der(14)add(14)(p11)x2 in most cells, long arm only painted, (Fig35s) has a DAPI-pale short arm.

15 3N, der(15)add(15)(p11), M2, (Fig35q) in most cells.

16 2N, del(16)(p11), one arm of a small metacentric (the DAPI pale arm), (Fig35r), whereas, the other is DAPI bright, M4, in all cells.

17 2N[5], 3N[4], 4N[1]

18 2N, del(18)(q21)x2 entirely painted, M5, (Fig.35u) in most cells.

19 2N, short arm of two chromosomes the size of an 8 or 10 (Fig.35l) in all cells.

20 4N, short arm of a long marker the size of 1p (Fig.35d) in nearly all cells.
21 2N, short arm of chromosome the size of an 11 x 2
ie.der(21)add(21)(p11)(Fig.35l) clearly 11q from the DAPI, in
all cells.

22 3-4N, small block on the proximal part of the long arm of an
acrocentric chromosome, smaller than a D,(Fig.35n),
der(22)add(22)(q11) in all cells.
Figure 35  Single colour FISH images from the XH1 cell line

(a) Paint 1 shows: 2N,

\[ \text{der(1)t(1;6)(p36;q21)} \ (\swarrow) \]

\[ \text{der(3)t(1;3)(p35;p14)} \ (\searrow) \]

\[ \text{der(20)t(1;20)(p12;p11)} \ (\swarrow) \]

b Idiogram illustration of cell above

\[ \text{der(1)} \]

\[ \text{der(3)} \]

\[ \text{der(20)} \]
(c) paint 3 shows: 3N, der(3)t(1;3)(p35;p14)

(d) paint 20 shows: 4N, der(20)t(1;20)(p12;p11)(arrow)
(e) paint 6 shows: 3N, der(1)t(1;6)(p36;q21)(large arrow)
der(6)t(6;10)(6p21->6q11::10q11->10qter)(small arrow)

(f) paint 10 shows: 3N, der(6)t(6;10)(6p21->6q11::10q11->10qter)
(arrow)
(g) paint 2 shows: 3N, der(7)t(2;7)(p15;p15)(arrow)

(h) paint 7 shows: 3N, der(7)t(2;7)(p15;p15)(M1)(arrow)

(i) paint 4 shows: 2N, del(4)(q21)(M3)(arrow)

(j) paint 5 shows: 5N, del(5)(q13)(M3)(arrow)
(k) paint 11 shows:
dup(11)(q14q22)x2(large arrows)
der(21)t(11;21)(q11;p11)x2
(small arrows)

(l) paint 21 shows: 1N,
der(21)t(11;21)(q11;p11)(arrow)

(m) paint 12 shows: 2N,
del(12)(p11)(small arrow)
der(22)t(12;22)(q13;q11)
(large arrow)

(n) paint 22 shows: 3N,
der(22)t(12;22)(q13;q11)(arrow)
(p) paint 13 shows: 1N,
der(13)t(13;13)(13q14→13q11::13p11→13qter)(large arrow)
der(15)t(13;15)(q21;p11)(medium arrow),
der(16)t(13;16)(q21;q10)(small arrow)

(q) paint 15 shows: 3N,
der(15)t(13;15)(q21;p11)(M2)(arrow)

(r) paint 16 shows: 2N,
der(16)t(13;16)(q21;q10)(M4)(large arrow), del(16)(p11)(small arrow)
(s) paint 14 shows: 2N, der(14)t(14;19)(p11;q11)x2 (arrows)

(t) paint 19 shows: 2N, der(14)t(14;19)(p11;q11)x2 (arrows)

(u) paint 18 shows: 2N, del(18)(q21)x2 (arrows)
The markers which could be identified as a result of the FISH painting were as follows:

**M1** = der(7)t(2;7)(p15;p15)

**M2** = der(15)t(13;15)(q21;p11)

**M3** = \( \text{del}(4)(21) \)

2 types \( \text{del}(5)(q13) \)

**M4** = der(16)t(13;16)(q21;q10)

**M5** = \( \text{del}(18)(q21)x2 \)

M6 which only occurred in some cells and at later passages was not identified.

Additions to chromosomes which could be identified were as follows:

Chromosome 1 der(1)t(1;6)(p36;q21)

Chromosome 3 der(3)t(1;3)(p35;p14)

Chromosome 11 dup(11)(q14q22)x2

Chromosome 11 der(21)t(11;21)(q11;p11)

Chromosome 13 der(13)t(13;13)(13q14->13q11::13p11->13qter)

Chromosome 14 der(14)t(14;19)(p11;q11)

The 10p<sup>-</sup> chromosome which only occurred at later passages was identified as a der(6)t(6;10)(6p21->6q11::10q11->10qter).
3.6.4. Clarification of breakpoint on chromosome 3 by dual colour FISH

In XH1, the green signal from paint 1 is seen on the der(3)t(1;3). The digoxygenin labelled 3p21 YAC was found on the normal chromosome 3, but no red signals were present on the der(3), so the breakpoint was proximal to the probe and put at 3p14(Figure 36).

Figure 36 Dual colour FISH image
chromosome 1 paint (green signal) and YAC 3p21(red signals) were co-hybridized to define the breakpoint on der(3)
As a result of all the G banding and FISH analysis, the karyotype can be written as follows:

\[ +\chi, 75 \leq 4n < XXXX, -1, \text{der}(1)t(1;6)(p36;q21) \leq -2, \]
\[ \text{der}(3)t(1;3)(p35;p14) \leq -4, \text{del}(4)(q21), +5, \text{del}(5)(q13)' \]
\[ \text{der}(6)t(6;10)(6p21->6q11::10q11->10qter), +\text{der}(7)t(2;7)(p15;p15)[6], \]
\[ -11, -11, \text{dup}(11)(q14q22)x2, -12, \text{del}(12)(p11), -13, -13, \]
\[ \text{der}(13)t(13;13)(13q14->13q11::13p11->13qter), \]
\[ \text{der}(14)t(14;19)(p11;q11)x2, \text{der}(15)t(13;15)(q21;p11), \text{del}(16)(p11), \]
\[ \text{der}(16)t(13;16)(q21;q10), \text{del}(18)(q21)x2, -19, -19, \]
\[ +\text{der}(20)t(1;20)(p12;p11), \text{der}(21)t(11;21)(q11;p11) \times 2, \]
\[ \text{der}(22)t(12;22)(q13;q11), [cP] \] (Fig. 37).
Figure 37 Revised karyotype of XH1 after FISH painting
3.7. Common rearrangements

The combined results from G banding, FISH painting, and FISH with cosmids and YACs has made it possible, in most cases, to locate chromosome breakpoints with some precision. Overall, about half of the breakpoints assigned with G banding alone had to be revised after FISH, and again the total number of breaks observed with G banding alone nearly doubled after FISH from 44 to 83 (Tables 6 & 7) as the markers were identified. There were 17 breakpoints in SM7, 14 in EH2, 13 in DE3, 7 in JE6, and 32 in XH1 (Table 7). The XH1 cell line appeared to have a more unstable karyotype than the others, which could account for the large number of breakpoints seen. This information provides a basis for comparing the independent breakpoints in all five cervical cancer cell lines.

3.7.1. Chromosomes most commonly involved

The frequency of involvement of each chromosome arm is shown in Table 8 and 9. Considering all five cervical tumour lines together, the chromosomes most commonly involved were 1 and 3 which were rearranged in all five lines, and chromosome 9 which was rearranged in four out of five (Table 7). Considering the chromosome arms separately, it can be seen that 1q is involved in all five, 9q in four out of five and 1p, 3q, 4q, 6q and 8q in three out of five (Table 8). This is reflected in the number of breakpoints (Table 9), although some lines such as XH1 had far more rearrangements than others, such as JE6, and this raised the overall number of breakpoints for some chromosomes such as 11 and 13 (Table 7). The total number of breakpoints for each chromosome arm is shown in Table 9 with 1q again topping the list with six breakpoints, followed by 1p, 6q and 13q with five, 3q, 9q and 11q with four and 4q and 8q with three each. The chromosome mentioned above will be considered individually.

3.7.1.1. Chromosome 1

A variety of chromosome 1 rearrangements were seen in most cell lines suggesting that this chromosome is constantly changing and evolving both in the tumour itself and in culture. Breaks at 1q10/11 were seen in three different tumour lines and a break at 1q21 occurred in
all three squamous carcinoma lines (Table 7 and 10). Isochromosomes for the long arm, i(1q) were found in two of the lines, DE3 and JE6 (Table 11).

3.7.1.2. Chromosome 3

Chromosome 3 rearrangements were again seen in all five tumour cell lines, two with short arm breaks found only in the adenocarcinoma (JE6) and adenosquamous (XH1) lines (Figure 30). The four long arm breaks in three different regions occurred only in the three squamous carcinoma lines (Figure 30 and Table 7), the two at 3q10 were seen as isochromosomes (Table 7). Chromosome 3 was involved in different rearrangements with four other chromosome arms, 1p, 2q, 4q and 5q (Figure 30). (Table 12)

3.7.1.3. Chromosome 4

Breaks in chromosome 4 occurred all in the long arm, at q35 and q33 in the squamous carcinoma lines EH2 & DE3, and q21 in the adenosquamous line XH1. No isochromosomes for the short arm i(4p) were found. (Table 7)

3.7.1.4. Chromosome 6

There were seven breaks altogether in chromosome 6, two in the short arm at p21 and p22, and five in the long arm; two at q11 and three at q21 (Tables 7 and 10) which could be important. Two of the 6q21 breaks occurred in the squamous lines SM7 and EH2 and the third was found in the adenosquamous line XH1.

3.7.1.5. Chromosome 8

Chromosome 8 rearrangements were only found in the squamous cell lines, all of which had an i(8q). The squamous line, SM7, also had a break at 8p10 in a rearrangement with 12q11.

3.7.1.6. Chromosome 9

Chromosome 9 was involved in four out of five lines, and at six different breakpoints, two in the short arm and four in the long arm. Aberrations included deletion in both arms, an i(9q) and a 9:9 translocation.
3.7.1.7. Other chromosomes

Chromosome 11 was only involved in two lines, EH2 and XH1, but in the latter it showed amplification of a region between 11q14 and 11q22 which could be a homogeneously stained region (HSR). There were four chromosomes with between two and four breakpoints, in which two of the breakpoints were in the same region. This is worth noting: 14p11, 15p11, 20p11 and 21p11 (Table 7 & 10).

3.7.2. Isochromosomes

The frequency of the different types of isochromosomes is shown in Table 11 with i(8q) as the most frequent followed by i(1q) and i(3q). The resulting overall p/q arm ratio was mostly around 3/5.

3.7.3. Types of rearrangements

In all, there was a wide variety of breakpoints within rearrangements, and apart from isochromosomes, no two translocations were identical (Table 12).

3.7.4. Amplification

FISH chromosome painting has revealed two potential sites for gene amplification in different lines; an extension of the 11q14 - q22 region in XH1 (Figure 28I) and double minutes in some cells of SM7 (Figure 20a) which showed as positive signals with the chromosome 1 paint (Figure15b)
<table>
<thead>
<tr>
<th>cell lines</th>
<th>Total breakpoints observed in more than two cells with G banding</th>
<th>Total breakpoints observed after FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM7</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>EH2</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>DE3</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>JE6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>XH1(pass35-39)</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>44</strong></td>
<td><strong>83</strong></td>
</tr>
</tbody>
</table>
Table 7  Chromosomes involved in rearrangements in more than two cells and independent breakpoints determined by G banding, and FISH, using Paints, YACs and Cosmids

$T^a$=Total breaks for each chromosome  $T^b$=Total breaks for each cell line

<table>
<thead>
<tr>
<th>Chr.</th>
<th>SM7</th>
<th>EH2</th>
<th>DE3</th>
<th>JE6</th>
<th>XH1</th>
<th>$T^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>q14</td>
<td>p22, q13</td>
<td>p11</td>
<td></td>
<td></td>
<td>4</td>
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<tr>
<td>1</td>
<td>p31, q21</td>
<td>q21</td>
<td>p10, q10, q21</td>
<td>q10</td>
<td>p36, p35, p12, q11</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>q11</td>
<td></td>
<td>q31</td>
<td>p15</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>q10, q26</td>
<td>q22</td>
<td>q10</td>
<td>p24</td>
<td>p14</td>
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</tr>
<tr>
<td>4</td>
<td>q35</td>
<td>q33</td>
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<td>5</td>
<td>p12</td>
<td></td>
<td>q31</td>
<td>q13</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>q11, q21</td>
<td>p22, q21</td>
<td></td>
<td>p21, q11, q21</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>q36</td>
<td>p15</td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>p10, q10</td>
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<td>q10</td>
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<td>4</td>
</tr>
<tr>
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<td>q10</td>
<td>p21, q12</td>
<td>p22, q33</td>
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<td></td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>p11</td>
<td></td>
<td></td>
<td>q11</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>q13</td>
<td></td>
<td>q11, q14, q22</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>q11</td>
<td></td>
<td></td>
<td>p11, q13</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>q12</td>
<td></td>
<td></td>
<td>p11, q11, q14, q21, q21</td>
<td>6</td>
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</tr>
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<td>q32</td>
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</tr>
<tr>
<td>15</td>
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<td></td>
<td>p11</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td>p13</td>
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<td>1</td>
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</tbody>
</table>

$T^b$  17  14  13  7  32  83
Table 8  Location of breakpoints in the different cell lines

<table>
<thead>
<tr>
<th>Chr arm</th>
<th>No of breakpoints</th>
<th>Total no of bps</th>
<th>No. of cell lines involved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SM7</td>
<td>EH2</td>
<td>DE3</td>
</tr>
<tr>
<td>Xp</td>
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<td></td>
</tr>
<tr>
<td>Xq</td>
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</tr>
<tr>
<td>1p</td>
<td>1</td>
<td></td>
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</tr>
<tr>
<td>1q</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2q</td>
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<tr>
<td>3p</td>
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<td></td>
<td>1</td>
</tr>
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<td></td>
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</tr>
<tr>
<td>4p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4q</td>
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</tr>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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<tr>
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<tr>
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Table 9  Total number of breakpoints in different regions

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Table 10  Frequency of recurring breakpoints

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Table 11  Frequency of isochromosomes

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Table 12  Translocation breakpoints in cervical tumour cell lines

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<td>8p10 --- 12q11</td>
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* Each chromosome translocation is shown twice (both ways round)
Figure 38  Idiogram representation of chromosome 3 with breakpoints

- **3p24 (JE6)** with **5q31**
- **3p14 (XH1)** with **1p35**
- **3q10 (SM7), (DE3)**
- **(EH2) 3q22** with **4q35**
- **(SM7) 3q26** with **2q11**

Chromosome 3
3.8. Comparative Genomic Hybridization

The CGH technique was applied to DNA samples from three of the cervical cell lines, a squamous carcinoma (SM7), an adenocarcinoma (JE6) and an adeno-squamous carcinoma (XH1), the karyotypes of which had already been extensively studied by FISH techniques, in order to test the sensitivity, reliability and reproducibility of CGH. It was also applied to a DNA sample from a fresh primary squamous cervical tumour, for which there was no cytogenetic information, in order to see how the result compared with the CGH findings on the cell lines.

Normal female DNA (diploid), extracted from a blood culture, was used as a control since all the cervical tumour samples were from female patients. Normal male metaphases were used to visualise the un-hybridised 'Y' at all times, in order to check that hybridization was occurring in the right place, since both control and test samples were female.

3.8.1. CGH on cultured cells

3.8.1.1. SM7

Co-hybridization of DNA from SM7 (green), and a normal female (red), onto a normal male metaphase is shown in Figure 40a. Five to twenty-five cells were examined using a Zeiss Axiophot fluorescence microscope which was attached to a cooled CCD camera and spreads were analysed by a computerised digital image analysis system using the Digital Scientific software "Smart Capture" programme. The green to red ratio of "n" (numbers of each chromosome examined) was presented as a 'profile' (Figure 41).

Figure 39 showed an enlarged single profile of chromosome 3 for explanatory purpose. The left hand side of the diagram is an idiogram of the individual chromosome so that regions of gain or loss can be mapped to particular bands on that particular chromosome. On the right is the profile. The middle 'black' line is a green to red ratio of '1' in the whole metaphase, which means that there have been equal amounts of hybridization of green and red probes. The inner red straight line is a green to red ratio of 0.75 (loss), and the outer red straight line is a green to red ratio of 0.5 (loss). Likewise, the inner green straight line is a green to red
ratio of 1.25 (gain) and the outer green straight line is a green to red ratio of 1.5 (gain/amplification). The central pink undulating line is the mean green to red ratio of 'n', the total number of chromosomes which have been observed in different metaphases of any one type. The two brown lines on each side of the pink line are the confidence limits of 95% (2SD). Figure 39 shows the profile for chromosome 3 from SM7, and n = 23, means that the computer has analysed 23 chromosomes 3, ie 12 cells have been examined, but in the 12th cell the computer was only able to examine one copy of chromosome 3 instead of the usual two.

**Figure 39** Enlarged single profile of chromosome 3 from SM7

![Enlarged single profile of chromosome 3 from SM7](image-url)
For most of the chromosome regions, the pink lines were on the middle black lines throughout their length, showing that their green to red ratios were 1. This means that the DNA copy numbers of both tumour and control for that particular region was in balance, and neither gain nor loss had occurred in these regions. For example, green to red ratios of 1 were seen on the chromosome arms 2p, 11p, 11q, 12p, 13q, 17p, 17q, 19p, 19q, 21q, 22q and Xp in SM7 (Figure 41).

DNA copy number gains in SM7 were obvious in 3q, 8q and 9q, where their respective pink lines were either close to, on, or beyond the first green line, showing a green to red ratio of around 1.25 (Figure 41).

Because of the previous detailed karyotyping, the CGH profiles (Figure 41) can be compared with the karyotype analysis for each chromosome, to see what CGH can tell us about the number of copies of any particular chromosome arm in the tumour cell line.

Table 13 shows the number of copies of each chromosome arm plus any additional regions obtained by G-banding and FISH, compared to the CGH profile for those particular regions. The copy numbers obtained by G banding and FISH are shown on the profile (Figure 41).

SM7 was a near tetraploid cell line (Histogram, Figure 12). Where there were four copies of a whole chromosome arm, the green : red ratio profiles from that particular region generally showed a score of 1, ie. chromosome arms 2p, 6p, 11p, 11q, 12p, 13q, 17p, 17q, 21q, 22q and Xp. However, on chromosome 7p, 7q, 9p, 10q, 14q, 16p, 16q 19p, 19q, all of which were also present as 4 copies, green :red ratio profiles showed a slight shift to the right of the mid line, ie. >1 (1.1) (Figure 41 & Table 13). In this case, a slight shift to the right, cannot be interpreted as a "gain".

Where there were three copies of a chromosome arm, the green : red ratio profiles score was 0.85 in most cases, ie 4p, 4q, 8p, 18p, 18q, and Xq. On chromosome arms 1p, 1q, 2q, and 3p the green : red ratio profiles did not go as far as 0.85, but fell just below 1,
showing a slight shift to the left of the black mid-line. Therefore, the overall score for three copies of chromosome arms in SM7 was clearly less than 1 and often as far as 0.85. Where there was a translocation which resulted in a change in copy number along a chromosome arm from 3 to 4 or the reverse, as in 6q and 15q respectively, there was clearly a shift in the profile mean which exactly matched the breakpoint (Figure 41 & Table 13).

For five copies, the green : red ratio profile lines showed an obvious gain as they were either on the 1.25 line, just below it, or around the 1.15 line. When the chromosome arms were present as six copies, the green : red ratio profile, was clearly on the 1.25 line or just above it (Figure 41 & Table 13).

On 3q, there were five copies of the whole chromosome arm, and due to the translocation, an additional copy of the region 3q26-3qter making the 3q26-3qter region equal to six copies. The green : red ratio profile for 3q26-3qter was above the 1.25 line. The same thing was seen in chromosome arms 8q and 9q, where the green : red ratio profile score was on or around 1.25 (Figure 41 & Table 13).

On 5p, there were five copies of the whole chromosome arm with an additional region from pter up to 5p12, making six copies of that region. The green : red ratio profile of chromosome 5 showed an obvious shift towards the 1.25 line, on 5p which was different from 5q where the profile lay on the 1.15 line (Figure 41 & Table 13).

The overall view of the profile showed narrow confidence limits for the green : red ratios on almost every chromosome (Figure 41). This means that the quality of the target metaphase was good and the quality of hybridization was even. The "n" numbers on the profile were between 19 & 24, showing that the computer digital image analyser was able to interpret high numbers of individual chromosomes in many cells on that particular slide (Figure 41).

Confidence limits (Figure 41) in the telomeric, centromeric and heterochromatic regions were wider, making it difficult to interpret data from these regions. However, confidence limits on the "Y" chromosome were very wide showing that no meaningful data could be gathered from this chromosome. Any hybridization to this chromosome must have been background,
since both cervical tumour DNA and female control DNA contained only XX chromosomal DNA. A male metaphase was deliberately used to keep the unhybridized 'Y' as a control for the technique to show that the right DNA is hybridizing with the correct target (Figure 41). In this tetraploid line, without karyotype information, it is not possible to assign a particular profile value to a given number of chromosomes of each of the types present. However, when hybridization is of good quality, as in this case, it is very clear which chromosomes are over-represented and which under-represented, and where the copy number changes within a chromosome or chromosome arm. CGH, therefore, gives a clear picture of where the breakpoints are occurring in aberrations resulting in uneven distribution of chromosome material.
Figure 40

a. CGH image of SM7

Green = Tumour DNA
Red = Control DNA

b. DAPI image of above metaphase
Figure 41  CGH profile of the SNP squamous cell line

The black numbers show the number of chromosomes represented in the karyotype (FISH-mapping + FISH).

Histone each chromosome region is a slide average.
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3.8.1.2. JE6

The JE6, adenocarcinoma cell line, was hyper-diploid (52), with the majority of chromosomes present as two copies, showing a profile of 1 (Figure 42, Table 14). However, the green : red ratio profiles of chromosome arms 1p & 16p were below 1 (0.85). The profiles of chromosome 7p, 7q, 12q, 16q, 17p, 17q and 22q also showed a shift to the left of the mid line but not as far as 0.85 (Figure 43 & Table 14).

Almost all the chromosome arms present as three copies, 2p, 9p, 10p, Xp, Xq scored 1.15. One exception to this was 10q although it could be argued that on closer examination the profile was to the right of the line rather than on top of it. The extra 20p is not detected but confidence limits of this chromosome are wide. There is clearly, however, an imbalance between p & q as the profile crosses the midline at the centromere. There is no clear explanation why the green : red ratio profile of 1q should be close to 1 despite there being four copies of this region present on account of the isochromosome 1q (Figure 43 & Table 14).

The region 2q11-31 was present as three copies whereas below this, 2q31-qter, there were only two. This was reflected in the profile which goes over the line in the 2q24-31 region and remains to the left of it down to the telomere (Figure 43 & Table 14).

The rearrangement between chromosome 3 and 5 was a reciprocal translocation so that the total chromosomal material representing chromosome 3 and 5 was in balance and equal to two copies each. In each case the profile goes down the midline (Figure 43 & Table 14).

On chromosome 9q, there was an additional region of 9q11-9q13 so that 9q11-9q13 was present as three copies. The profile for the region scored >1 going back to 1 for the rest of 9q (Figure 43 & Table 14).

CGH profiles of this cell line, therefore, generally reflect the chromosome numbers present, but losses might have been suspected for some chromosomes without accompanying karyotype information. Most of the over-represented chromosome regions were seen on the profiles, and as expected, the reciprocal translocation was not detectable.
Figure 42

CGH image of JE6

Green = Tumour DNA
Red = Control DNA

DAPI image of above metaphase
Figure 43 CGH profile of the JE6 adenocarcinoma
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</table>
3.8.1.3. XH1

A CGH image from XH1 DNA is shown in Figure 44, with the modified DAPI image below. The profiles for each chromosome are illustrated in Figure 45.

The confidence limits of the profiles of the XH1, tetraploid cell line, were, on the whole, wider than those of the equivalent tetraploid line SM7. They also became wider at heterochromatic regions and telomeres, so that ratios in these regions had to be disregarded. Unlike SM7, profiles did not distinguish between 3 & 4 copies of whole chromosomes which were all scored as 1 or just below. Profiles where 5 & 6 copies of whole chromosomes were present also showed a green : red ratio of 1 (Figure 45 & Table 15).

Nevertheless, where there were changes of copy number within a chromosome (Table 15) and a shift in the ratio profile from one end of the chromosome to the other, the shift was nearly always in the right direction, ie. chromosomes 1, 2, 3, 4, 10, 11, 13, 18 & 19 (Figure 45), the one exception being chromosome 7. This must indicate that although the hybridization was not ideal, it must be giving us some information on where the breakpoints occurred (Figure 45 & Table 15).

The amplification in the 11q14-22 region was clearly visible as a dramatic change in the DNA profile not seen in any other chromosome (Figures 44, 45, 46 & 47) (Table 15). On the profile for chromosome 11, the loss or deletion at 11q23 was seen (Figure 45), but not as clear as on the colour image in Figure 44.
Figure 44

CGH image of XH1

Green = Tumour DNA  Red = Control DNA

DAPI image of above metaphase

Chr 11
Figure 45  CGH profile of the XH1 adenosquamous cell line

Case: XH  Slide: 1  Cell: 23  Patient:
### Table 15

**XH1 comparative data from G banding/FISH painting and CGH**

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<th>chr arm</th>
<th>no of whole arms</th>
<th>additional regions</th>
<th>region of change</th>
<th>green : red</th>
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Figure 46  Enlarged chromosome 11 from XH1

(a) CGH image of Chromosome 11 from XH1 showing the amplified region 11q14-22

(b) G banded chromosome 11

(c) Pseudo-colour image of chromosome 11 showing amplification, 11q14-22 as a prominent green band and deletion as a predominant red region 11q23 below the amplification.
Figure 47  Idiogram of chromosome 11 showing amplification 11q14-22 and deletion 11q23

Chromosome 11
3.8.2 Primary squamous cervical tumour, uncultured

CGH images and profiles for the uncultured primary tumour are shown in Figure 48 and 49, with losses and gains detailed in Table 16. Obvious profile changes not associated with centromeres and telomeres are listed below:

1) 2q14-q21 possible region of amplification

2) 3q10 switch from 0.85-1.25
   3q24 switch from 1.25-1.00

3) 6q10 switch from 1-0.85

4) 8q10 switch from 1.15-1.25

5) 10p11 switch from 0.6-0.85

6) 11q21 switch from 1.15-1.00

7) 13q21 switch from 0.75-1.00

8) 18q11 switch from 1.25-1.00

Most of the above involve a change in green:red ratio of 0.15 or more and probably indicate breakpoint sites.
Figure 48  CGH image of the primary tumour

CGH image of uncultured primary cervical squamous tumour

Green = Tumour DNA  Red = Control DNA

DAPI image of the above metaphase

3q  3q
Table 16
CGH data from the uncultured primary tumour

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3.8.3 Comparison of profiles in the SM7 and the primary tumour

CGH profiles of an uncultured fresh primary cervical squamous carcinoma showed some similarities and differences in comparison with SM7. The profiles of chromosome arms such as 3q, 8q, 9q, 20p, 20q, and Xq showed similarities to SM7. The gain of 3q in SM7 was the whole arm, whereas in the uncultured primary tumour, the 3q gain was on 3q11-25 (Figure 50 and Table 17). Both 8q and 9q showed similar profiles of gain in the primary tumour and SM7. For chromosome 20 the primary tumour showed a much larger gain for both p & q, far more than in SM7 (1.5 and 1.15 respectively). Chromosome Xq showed losses in both the primary tumour and SM7 (0.85) (Figure 50) (Table 16 & 17).

Reversed profiles were seen for 5q, 8p, and 10p. 5q showed a gain in SM7 (1.15) but a loss in the primary tumour (0.85). Chromosome 8 short arm (8p) showed a loss in SM7 (0.85) and a gain in the primary tumour (1.15). 10p showed a gain in SM7 (1.15) but a loss in the primary tumour (0.5) (Figure 50 and Table 16 & 17).

Differences between the primary tumour and SM7 were seen in 4p, 4q, 5p, 6q, 7p, 7q, 9p, 10q, 11p, 11q, 12q, 14q, 16p, 16q, 18p, 18q, 19p, and 19q. Gains of 7p & q (1.25), 9p, 11p & q, 14q, 16p & q, and 19p & q (1.15), were only seen in the primary tumour and not in SM7. Loss of 4p & q, 18p & q (0.85), and gain of 5p (1.15), were only seen in SM7 and not in the primary tumour (Figure 50 and Tables 16 & 17).

The probable breakpoint sites found in the primary tumour by CGH were compared to the breakpoints found by conventional cytogenetics in the five cultured cell lines (Table 7). These are shown in Table 18.
Figure 50  Comparison of SM7 and primary tumour profiles

Case: SM  Slide: 1  Cell: 23  Patient:  

primary tumour profiles
Table 17 Comparison of CGH gains and losses in SM7 and primary tumour

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<th>squamous cell line</th>
<th>primary tumour</th>
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<tr>
<td>Xp</td>
<td>q</td>
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Regions of gain common to both samples are highlighted
Regions of loss common to both samples are shown as *
Tables 18  Probable breakpoints in the primary tumour by CGH compared to those seen in the five cultured cell lines by FISH/ G banding

<table>
<thead>
<tr>
<th>Probable breakpoints in the uncultured primary tumour by CGH</th>
<th>Breakpoints in the same arms in cultured cell lines by FISH/ G banding</th>
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<tbody>
<tr>
<td>2q14</td>
<td>SM7 (q11)</td>
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<td>2q21</td>
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<td>3q10</td>
<td>SM7, DE3 (q10)</td>
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<td>3q24</td>
<td>SM7 (q26), EH2 (q22)</td>
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<tr>
<td>6q10</td>
<td>SM7, XH1 (q11)</td>
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<td></td>
<td>SM7, EH2 &amp; XH1 (q21)</td>
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<td>8q10</td>
<td>SM7, EH2 &amp; DE3 (q10)</td>
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<td>SM7p11</td>
</tr>
<tr>
<td>11q21</td>
<td>EH2 (q13), XH1 (q11, q14, q22)</td>
</tr>
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<td>SM7 (q12), XH1 (q11, q14, q21)</td>
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<td>18q11</td>
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Chapter 4

Discussion
General

Conventional banding techniques are able to describe chromosome abnormalities in near-diploid malignancies, with a high level of accuracy where there are simple rearrangements. As modal numbers and chromosome rearrangements increase, the task becomes much more difficult, leaving many markers and additions unidentifiable. With the advent of FISH and the development of whole chromosome painting probes, complicated karyotypes can now be resolved much more accurately.

Establishing long-term cell cultures from tumour samples is not easy, and may take many cell doublings during which in-vitro karyotype modification can occur. Once the cells are growing well, good quality cytogenetic preparations can be made repeatedly, so that full advantage can be taken of all the new FISH technology, to obtain accurate cytogenetic information. Bearing in mind that some of the changes seen in cell cultures may have arisen in vitro, newly established cell lines are particularly useful for certain tumour types, such as cervical cancer, where there is a shortage of karyotype information.

It was clear from the outset, when we first tried to establish a karyotype for these cell lines, that FISH would be extremely useful. For the hypertriploid / tetraploid squamous carcinoma cell lines, SM7, EH2 and DE3, conventional G banding left many questions unanswered. Nearly all of these were resolved using the chromosome-specific paint probes. Dual colour FISH with selected paints was then used to confirm certain translocations, and YACs & cosmids to refine the breakpoints. With a more simple karyotype, such as JE6, the FISH was largely confirmatory, but still added useful information, so that more valid comparisons between the cell lines could be made.

As in some other studies (Ruess et al., 1993), identification of abnormal chromosomes by G banding was not always confirmed by chromosome painting, and likewise, chromosomal abnormalities detected by chromosome painting were not always seen initially in G banded chromosomes. This was probably caused by the poor resolution of G banding and by the inadequate quality of DNA probes. Chromosome painting with complementary conventional
G banding can detect chromosome fragments of more than about 5 Mb (Ruess et al., 1993) providing good resolution of even quite small rearrangements. FISH with YACs and Cosmids has been particularly useful in this study, in refining the position of doubtful breakpoints, especially on chromosome 3.

Obviously it is much better to look at genetic changes in tumour material without an intervening culture period. However, even using FISH, it is difficult to achieve anything approaching the degree of resolution mentioned above in chromosome preparations from fresh tumour material, owing largely to the poor quality and scarcity of metaphases, particularly where the chromosome number may be large and the karyotype complicated. CGH, which uses genomic tumour DNA, hybridised to good quality normal chromosomes, is the ideal technique for looking at karyotype changes in tumours, particularly where there is a low success rate in obtaining good quality metaphases. As mentioned previously, although it poses certain other disadvantages, CGH makes it possible to screen genetic changes in the entire tumour genome in a single experiment (Arnold et al., 1996). This technique, which has now been used to study more than 1500 tumours, 75% of them solid tumours (Kallioniemi O-P, 1996), has been tested in the present study on DNA from cultured cervical tumour material. This material has been accurately karyotyped, in order to obtain a direct comparison between the two techniques. By this method, the efficacy of CGH in picking up both numerical and structural aberrations can be tested, and these results can then be compared with CGH information obtained directly from fresh cervical tumour DNA.
Information from conventional karyotyping using G banding and FISH

Chromosome 1

In this study, the highest number of breakpoints occurred in chromosome 1. The breakpoints on the short arm occurred along the whole length from 1p36 to 1p10. On the long arm, breaks were at 1q10/11 in three lines, a squamous, an adeno-squamous and an adenocarcinoma, and at 1q21 in all three squamous lines.

Double minutes were found initially in some cells from one squamous line, SM7, and FISH painting showed that they originated from chromosome 1. It was not possible to locate the exact region of amplification, as by the time CGH was performed on this culture, there were only a very few cells with double minutes. Genes on this chromosome are thought to have an important role in cell immortalisation (Suga et al., 1990). There is a possibility that genes on chromosome 1 are disrupted by integration of viral sequences from human papilloma viruses (Popescu et al., 1992), or from herpes simplex viruses (De Braekeleer et al., 1992), both of which have been associated with cervical carcinoma. Epithelial cells immortalized in vitro with HPV show alterations of chromosome 1 before they become tumourigenic, suggesting that these changes could be early genetic events in cervical neoplasia (Popescu et al., 1992). Deletion of tumour suppressor genes and activation of oncogenes could follow as the tumour develops.

In 1996, Vojta et al. also demonstrated the role of alterations of human chromosome 1 in cellular immortalization. In addition to the candidate region for the senescence genes on 1q observed by previous studies, these authors suggested that there is a second senescence gene on 1q31-1qter. Sequence-tagged site analysis allowed the interval which contains the second chromosome 1 senescence locus, previously located by Suga et al. in 1990 at 1q23-1qter, to be narrowed down to 1q31-1qter. Their observations showed two regions of loss of 1q material, between 1q42.1-42.3 and 1q42.3-1q43, suggesting that the distal 1q senescence gene(s) localise within 1q42-43.
Chromosome 3

Chromosome 3 was rearranged in all five cell lines. In spite of investigations with YACs and cosmids, in addition to the conventional banding and FISH painting, no consistent breakpoint occurred in all the lines. However, the breakpoints were confined to the long arm in the three squamous cell lines, SM7, EH2 and DE3, and were located only on the short arm in the adenocarcinoma line, JE6, and in the adeno-squamous carcinoma line, XH1. All three squamous lines showed a gain of all or part of the long arm of chromosome 3. Isochromosomes for the long arm and the breaks in the short arm could have resulted in loss of heterozygosity for loci on 3p, which has been reported in up to 70% of cervical cancer by several groups (Table 1 and 1.2.8.2.).

Rearrangements of chromosome 3 are common in HPV immortalised human cell lines (Smith et al., 1989), but it may be necessary for specific translocations involving oncogenes such as ETS 2 on 21q22 to occur, before cells become tumourigenic (Dyer Montgomery et al., 1995). In 1995 Popescu et al. showed that cervical cells, transformed in vitro with HPV, only had chromosome 3 abnormalities when they became tumourigenic, after transfection with another oncogene H-ras. On the other hand, rearrangements of chromosome 3 are also found in HPV negative cervical carcinoma cell lines such as HT-3 (Zimonjic et al., 1995). A similar situation was found in JE6, the only HPV negative line in this study, which has a very stable hyper-diploid karyotype with only six rearrangements, including a 3q translocation. In 1994, Mitra et al. reported breakpoints in 3q in two newly established cervical cancer cell lines in which the HPV status were not defined. Clearly, disturbances of genetic material in chromosome 3 are an important factor in cervical cancer, whether or not the HPV genome is involved.

Use of CGH demonstrated that in SM7 a chromosome 3q gain was again quite obvious. This was also reproduced in an uncultured fresh primary squamous carcinoma sample. Similar findings were published in 1996 by Heselmeyer and co-workers using CGH applied to normal cervical epithelium, to cervical samples with CIN I, II, III and to micro-invasive carcinomas. In CIN III only 10% of specimens showed a 3q gain, whereas it was
seen in 90% of invasive squamous carcinoma samples. The authors suggest that a 3q gain could be the pivotal genetic aberration at the point of transition from severe dysplasia to invasive cervical carcinoma.

Chromosomes 4 and 5

In this present investigation, small metacentric chromosomes, superficially resembling isochromosomes, were found in one squamous carcinoma cell line (SM7) and in the adeno-squamous carcinoma cell line, XH1. FISH showed that none of these were isochromosomes, but SM7 carried a t(X;5), and XH1 a del(4)(q21) and a del(5)(q13).

In 1982, a small metacentric chromosome, thought to be 5q-, was found in five out of nine cervical carcinoma samples by Atkin et al. In 1984, the same group observed a small metacentric chromosome, thought to be either a chromosome 4 or 5, long arm deletion, or a short arm isochromosome, often in duplicate among 10 cases of cervical carcinoma samples. In 1990, Atkin and co-workers found i(5p) or i(4p), in as many as 77% of 43 cases of cervical cancer. Although Sreekantaiah et al. in 1991 did not find i(5p) in their large series of 150 cervical tumour samples, Mitra and co-workers in 1994 found i(5p) in two newly established cervical squamous carcinoma lines. Ruess et al. in 1993 also confirmed an i(5p) (m11) in the HeLa D98 studied by FISH chromosome painting. In the present study, it is possible that i(5p) could have been lost during cell cultivation, but specific isochromosomes like the i(12p) found in testicular tumours are usually retained (Parrington et al., 1994).

In 1990 Ning et al. observed the reversion of Hela cells to a senescent phenotype upon microcell-mediated transfer of a single copy of human chromosome 4, and the authors suggest that there may be a putative cervical cancer tumour suppressor gene(s) on chromosome 4. This year (1996) Hampton et al. published their observations of LOH on chromosome 4 in 58 cervical carcinoma samples. They used a fluorescence-based detection system with computer software which was able to perform automated quantitation and
calculation of allelic ratios at multiple micro-satellite loci per gel lane. This increased throughput, accuracy and reproducibility of LOH determination. The data suggested that there were two independent tumour suppressor genes which mapped to the distal part of 4p and 4q in cervical cancer. Results on 4q agreed with the study by Mitra et al. (1994), using conventional allelotype analysis, but Mitra et al. did not show LOH on 4p by Southern blotting using a probe mapped to 4p16, whereas Hampton et al. showed a frequency of 36% on 4p using their method. Karyotype analysis of near-tetraploid cell lines in the present study show that chromosome 4 was consistently under-represented. Breakpoints in three of the lines were all in the long arm, 4q35, q33 and q21.

A high frequency of allelic loss in the short arm of chromosome 5 has been shown in the past in cervical tumours, suggesting that there has been loss of a candidate tumour suppressor gene located on 5p. Mitra and co-workers studied 19 cases of CIN, including 5 cases of CIN III, and 46 untreated invasive cervical carcinoma samples, by PCR using five polymorphic micro-satellite loci mapped to 5p. The study showed that LOH was seen in 25 out of 45 informative invasive carcinomas (55.6%), one of five cases of CIN III (20%) and three of 14 other CIN grades (21%). Among the five loci tested, 5p15.1-15.2 exhibited LOH in 12 of 25 cases of invasive carcinomas (48%), one of the three cases of CIN III (33%) and three of five other grades of CIN (60%), suggesting this to be the site on 5p of a novel candidate tumour suppressor gene. In addition, in the same study, among the 12 cases which showed micro-satellite instability, eight (66.7%) had instability affecting 5p15.1-15.2. The observations of allelic loss and micro-satellite instability in the region of 5p15 suggested that genes located here may play a role early in the development of cervical cancer. No breakpoints involving the region 5p15 were found in any of the five cell lines (Table 7) in the present study. One occurred at 5p12 and two were in the long arm.
Chromosome 6

A del(6q) very similar to that described by Mitra et al. (1994) was seen in one of our cell lines, SM7, but in this case the breakpoint was nearer to the centromere (6q11). Another breakpoint, however, was found in three of our cell lines, SM7, EH2 & XH1, further down the long arm at 6q21, within the common region of deletion described by Mitra in 1993.

A recent LOH study which investigated loci on all autosomal arms in cervical cancer (Mullokandov et al., 1996) has shown that the frequency of LOH on 6p (43%) was even higher than at 3p (39%). LOH on 6q showed a lower frequency, but still above 20%. Hence tumour suppressor genes on this chromosome may also be important in the development of cervical cancer.

Chromosome 8

In this study, i(8q) was found in three squamous cell lines, all of which carried HPV DNA. There were no breaks on chromosome 8 in XH1 (HPV 16 +ve) and in the only HPV-negative cell line, JE6. None of the cell lines had breakpoints at 8q22, the HPV 18 integration site found by Gallego et al. (1994) or at 8q24, close to the myc oncogene, another region suggested to be a preferential target site for HPV integration (Couturier J et al., 1991). Although the exact integration site in our cell lines has yet to be established, the 8q;12q translocation in SM7 was very similar to that described by Gallego et al. in 1994, in which they demonstrate HPV integration at 8q22.1. If HPV DNA was integrated into 8q in the three squamous lines described here, the extra copies provided by isochromosome formation may be sufficient to induce the transformed state.
Chromosome 9

In this study, chromosome 9 abnormalities were seen in four out of five of the cell lines. Two had breakpoints in 9p and four in 9q. This chromosome does not show high levels of LOH in cervical cancer in studies by Mitra et al. in 1994 and by Mullokandov et al. in 1996, but the breakpoints in our cell lines were in the region of the multiple tumour suppressor gene (MTS-1) at 9p21-22 (Kamb et al., 1994), more proximal to those described by Zimonjic et al. (1995) at 9p24 in two HPV negative cervical carcinoma cell lines, where extensive rearrangement of chromosome 9 was seen.

Chromosome 11

LOH is quite frequent on chromosome 11 (Mullokandov et al., 1996), and 11q structural changes have been seen frequently in direct preparations of cervical tumours, reported by Atkin et al. in 1990. In addition, the HeLa tumour suppressor gene was thought to be located on this chromosome. However, in this study, we found 11q breakpoints in only two of the cell lines, EH2 and XH1. In XH1, the marker was composed entirely of chromosome 11 material, suggesting amplification of DNA in the 11q14-22 region. This was confirmed by CGH which also showed a deletion in 11q23. This observation supports the findings of Hampton et al. (1994), and also their suggestion that a tumour suppressor gene relevant to cervical cancer may possibly map to 11q22-24.

EH2 had a breakpoint on 11q13, which is the region where the PRAD1 gene encoding Cyclin D1 is located (Komatsu, 1994). The PRAD1 has been shown to be over-expressed in cells with 11q13 amplification. Amplification in XH1, thought to be at 11q14-22, is not far from this site. PRAD1 complexes to the product of the retinoblastoma tumour suppressor gene, and inactivates this gene. Likewise, proteins derived from HPV also complex with the retinoblastoma gene. A study by Kurzrock et al. (1996) suggested that PRAD1 deregulation may be a significant molecular event in gynaecological squamous cancer including of cervical origin.
Chromosome 17

Chromosome 17p+ was found in 2 out of 9 cervical carcinoma samples in 1982 (Atkin et al.), and chromosome 17 abnormalities were also found in 4 out of 10 cases in 1984 by the same authors (Atkin et al.). In 1990 Atkin et al. reported again that chromosome 17 abnormalities were seen in 47% of the 43 cervical carcinoma samples they investigated. In 1994, Atkin et al. demonstrated two identical findings of rearranged chromosome 17 in two squamous carcinoma cell lines, in which the whole 17p had been replaced by the long arm of chromosome 22 (17;22). The authors suggested that these chromosome rearrangements may be significant in cervical cancer development. In the present study, however, only one squamous carcinoma cell line (EH2) had chromosome 17 involvement, (t(6;17)).

Comparison with HeLa D98/AH-2

To date, the only other study which has used FISH painting to describe rearrangements in detail in cervical cancer cell lines is the report by Ruess et al. in 1993, on the HeLa line D98/AH-2. This line has been in culture for a great many years, and the karyotype described has certainly changed a great deal in vitro. Nevertheless, it is worth noting that five breakpoints in the HeLa line were the same as those reported here; 12q11(SM7), 11q13 (EH2), 5q31(JE6) and 5q13 and 13q21 (XH1), but none of these occurred more than once.
Conventional karyotyping showed that SM7 was a near-tetraploid line. When there were four copies of a whole chromosome arm, the green : red ratios were close to 1, which demonstrates that a diploid sample can be used as a normal control to co-hybridize with any test sample, irrespective of its modal number, because of the proportional representation of every chromosome arm and region in both samples, when equal quantities of normal and tumour DNA are hybridized onto normal target metaphases. When copy number changes are being assessed in a near-diploid sample, a gain of a whole chromosome in half the cells analysed will give a profile around 1.25 (Schrock et al., 1996). When a near-tetraploid sample is being analysed, the proportional increase or decrease will be smaller, i.e., an increase from 4 to 5 copies would change the ratio by 25% or less, depending on what proportion of cells showed the change. For instance, the profile for chromosome 2 (Figure 41) showed that the green : red ratio for the short arm lay along the mean black line, a value of 1, but for 2q it fell to a value of 0.9. Karyotyping showed that the copy number fell from 4 to 3 at the centromere, and this was reflected in a small shift of 0.1 in the green : red ratio. This shows that small changes in ratio in tetraploid tumours do reflect underlying karyotype changes as long as they are consistent along the length of the chromosome arms. Such profile shifts, caused by changes in copy number in different regions along the same chromosome, allow us to work out the possible breakpoints where there is no karyotype information.

Similarly, in SM7, the chromosome arms with five copies showed a certain degree of gain compared to the chromosome arms with four copies (10p->10q and 12q->12p). However, the degree of gain varied from one region to another despite all of them having five copies. For instance, 5q and 20q, had green : red ratios of 1.15 and 1.1 respectively, whereas the green to red ratio profile for 3q was even further to the right (1.25). It is possible that the DNA sequence gain in 3q could have been much more than in 5q, 12q or 20q, all of which
had a chromosome copy number of five. CGH could then not only indicate chromosome copy number changes along the length of a chromosome, but also provide information on DNA sequence number changes where the chromosome copy number remains the same. In this study the three isochromosomes i(3q), i(8q) and i(9q) present in this cell line all showed up clearly on the CGH profiles.

In SM7, the profile showed generally narrow confidence limits, except in the telomeric, centromeric, acrocentric and heterochromatic regions. The number of individual chromosomes that the computer digital image system was able to analyse was also high (n = 12 - 24, ie. 6 - 12 cells). The narrow confidence limits and high 'n' number for almost every chromosome in the genome indicates that the CGH results are reliable on account of the good quality of both tumour and control DNA, the quality labelling, the hybridization and the target metaphases. The longer the DNA sequences obtained at the time of nick translation, the better the hybridization will be, giving a less granular and more even digital image for analysis.

In contrast, the confidence limits for the Y chromosome were very wide (Figure 41) showing only background hybridization on the Y, since neither SM7 DNA nor female control DNA contained any Y material (Figure 40).

In conclusion CGH analysis gave a clear indication of the chromosome copy number changes found in conventional cytogenetics, and showed over-representation of 3q, 5p, 8q, 10p, 12q and 20. Under-represented regions included 2q, 3p, 4, part of 6q, 8p, part of 15q, 18 and.
Conventional karyotyping showed a hyper-diploid line which was co-hybridized with normal diploid DNA. Most chromosome arms or regions with two copies gave a green: red ratio profile of 1. This was also seen in 3p and q (a balanced translocation), as expected. However, this did not seem to be the case for chromosome 1, where there were two copies of 1p and four copies of 1q. The profile showed a change from 0.85 to 1 from p to q, and a smaller change than expected for a doubling of the copy number. This is difficult to explain, as four copies of 1q should show a ratio of 1.5.

Most other chromosomes where the copy number changed from 3 to 2 along the length, such as 2 and 9, gave a clear profile shift at the breakpoints. The exception was chromosome 20 where no change was detectable, but good hybridization is known to be a problem in G-light bands, and this is reflected in wide confidence limits.

The DNA sequence gain on 1q was quite obvious on visual assessment of the CGH colour image (Figure 42). There is the possibility that fluorescent signal loss occurred during the six month period from the first image analysis of CGH slides immediately after the experiments, and the generation of profiles on these images six months later. At the time of profile generation, it was not possible to use the original images, captured six months earlier, because of a difference in the computer soft-ware system. Immediately after the experiments, images were analysed and captured using the 'Smart Capture' soft-ware programme from Digital Science, Cambridge. Six months later, 'Cytovision' soft-ware, from Applied Imaging, was used to recapture the images from the original slides to generate green: red ratio profiles. All CGH slides were stored together at the same temperature of 4°C, so that CGH slides from SM7 should have shown a similar deterioration in the fluorescent signal as the JE6 slides.

For JE6, the number of chromosomes analysed ranged from 13-21 among the autosomes, that is from 7-11 cells. For sex chromosomes, only 7 copies of X were analysed in 4
cells, and yet the green : red ratio profile was meaningful. The unexpected profile for 1q was therefore not due to the low number analysed.

In conclusion, although CGH did not give an accurate copy number for chromosome 1, analysis of this hyper-diploid line reflected three out of the four copy number changes within a chromosome found by conventional cytogenetics.

XH1

Conventional karyotyping showed that this was also a near-tetraploid line. The computer digital image analysis examined 20-28 copies of each of autosome (10-20 cells), but the X chromosome was analysable in only 6 cells. Almost all of the chromosome arms or regions with four copies showed green : red ratio profiles along the mean black line, as expected. Ratios along 13q, 19q and 20p, however, showed profiles greater than 1, despite there being four copies of each. In these 3 cases, regions were not long enough for a reliable interpretation, and they were also too close to acrocentric, centromeric and telomeric ends.

There was also a problem with the green : red ratio profiles of chromosome arms with three copies, where most of these did not show a ratio lower than 1. Chromosome 1q was an exception, since its green : red ratio profile was just to the left of the mean black line. Chromosome 16 (3 copies), also had a profile lower than 1, although its green : red ratio was difficult to interpret once the telomeric and centromeric regions had been deducted from the profile. Most chromosome regions with five or six copies did not show a gain as expected, but clear switches were seen in chromosomes 1, 2, 3, 4, 13q, 18 and 19, all in the expected direction. However, some, such as in chromosome 7, were notably in the wrong direction, so that accurate breakpoints could not have been predicted from these profiles, as they were in SM7.

Amplification at 11q14-22, however, remains obvious, and even the loss / deletion at 11q23 was seen, although not as strongly as the amplification. The del 11q23 was more
prominent in the CGH colour image of XH1 in Figure 44, which was captured six months earlier, than the profile shown in Figure 45.

Uncultured primary tumour

Since it was not karyotyped beforehand, the modal number of this tumour was unknown, so it was impossible to speculate on the copy numbers of individual chromosomes. Copy number assessment for individual chromosomes in the un-karyotyped sample, based on CGH profile shifts (gains / losses), is only possible when the copy number of some of the chromosomes has been obtained by other molecular techniques, such as interphase cytogenetics. Speicher et al. in 1994 reported that data from CGH was found to have a linear correlation with the average signal number in interphase nuclei. Confidence limits for these profiles were generally quite wide, showing that hybridization of tumour and normal DNA was not as good as in SM7, perhaps an account of sub-optimal DNA quality, sub-optimal length of DNA sequences at the time of nick translation, or poor quality of target chromosomes. It is quite difficult to avoid necrotic areas or adjacent normal tissue at the time of sampling from tumour material for DNA extraction. Centromeic regions of chromosome 1, 9 and 16, and acrocentric regions of 13, 14, 15 and 20 showed inadequate blocking of repeat sequences by Cot-1 DNA, although the same protocol was followed for each cell line.

In regions away from these areas, however, there were clear switches in ratio value, which could be interpreted as possible breakpoints, such as at 3q10 and 8q10, which were also found in the squamous cell lines. The only small region of possible amplification was at 2q14-21.

As a result of the molecular cytogenetic investigations which have been carried out here on material from cervical tumours using G banding, FISH and CGH, a number of interesting conclusions can be drawn.
The number of chromosomes for the three newly established cell lines from squamous cell carcinomas of the cervix, the most common cervical tumour, and from adeno-squamous carcinoma was always in the hyper-triploid / tetraploid range, with relatively few chromosome rearrangements which could easily be identified using FISH. This is very similar to the in vivo picture found by Heselmeyer et al. (1996) in biopsies of cervical tumours. They showed that tetraploidization was the first genetic abnormality which occurred early at the mildly dysplastic stage. HPV sequences and chromosome abnormalities were detected after this at the severe dysplasia stage, and finally, over-representation of 3q was the predominant abnormality at the invasive stage.

When CGH was conducted in this investigation on cell lines which had previously been thoroughly karyotyped, it was clear that the profiles, although they could not give accurate copy numbers of whole chromosomes, could indicate over or under-representation of whole arms or regions, so that regions where the profile changed marked the positions of breakpoints. Consistent and specific switches identified by this method in fresh tumour material would indicate regions which might harbour oncogenes or tumour suppressor genes, and which would merit further molecular investigation.

CGH cannot detect tumour suppressor gene inactivation if it is caused by mechanisms other than physical loss. Neither can it pick up oncogene activation caused by point mutation, transcriptional gene activation or by chromosomal translocation (Arnold et al., 1996). However, it facilitates the location of chromosome regions of 'amplification' or 'deletion', which might harbour potential 'oncogenes' or 'tumour suppressor genes' respectively. In this study, CGH has confirmed the amplification in XH1 at 11q14-22 and revealed a deletion immediately distal to this at 11q23. One could hypothesise that loss of a tumour suppressor gene, resulting from the deletion at 11q23, could lead to over-expression of an oncogene in the vicinity, which would be amplified because of the loss of a crucial suppression mechanism. Although this was only found in one tumour cell line, it was present at a very early passage level, and in view of other abnormal findings in cervical cancer in this region, it may harbour genes which are important in the oncogenesis of some cervical tumours.
CGH can also reveal, in tumours, several novel regions of frequent DNA sequence gain or loss, which were previously unknown. These regions could be the sites of currently unknown genes with a significant role in the mechanism of neoplasia (Szymanska et al., 1996). In this study we did not find any new regions, since the number of tumour and cell line samples analysed by CGH was very small. In the future, two more cervical squamous cell lines, EH2 and DE3, will be examined with CGH as well as a series of uncultured fresh cervical tumour samples.

CGH on a large number of tumour samples, of a particular tumour type, will allow identification of any consistent recurring chromosomal imbalances which could eventually lead to the identification of candidate genes, or chromosomal regions of biological importance, for that tumour. Even in a genetically complex tumour where almost every chromosome arm is involved in DNA sequence copy number changes, aberrations are clustered at certain chromosome regions and indicate that these changes are likely to be significant in tumourigenesis (Arnold et al 1996). These objectives have been clearly demonstrated in the various publications on CGH which have now been applied to a number of different tumours, such as cervical (Heselmeyer et al., 1996), ovarian (Arnold et al., 1996), colorectal (Ried et al., 1996), astrocytomas (Schrock et al., 1996), liposarcomas (Szymanska et al., 1996), gliomas (Muleris et al., 1994), uveal melanomas (Speicher, 1994), prostate (Cher et al., 1994) and breast tumours (Kallioniemi et al., 1994).

CGH can also be used on DNA extracted from formalin-fixed material, which enables a correlation to be made between CGH observations, histology and the clinical outcome, by a retrospective analysis of archival materials (Ried et al., 1996). CGH is a suitable experimental approach for visualising chromosomal gains and losses in histologically characterised serial tissues, such as: normal epithelium, low and high grade benign tumours and invasive malignant tumours of the same origin, in order to study different chromosomal aberrations at different stages of tumour progression. The initial genetic disturbance in cervical carcinogenesis is a tetraploidization without aneuploidy, which is
found in mild and moderate dysplasia, whereas in contrast, severe aneuploidy was observed even in low-grade colorectal adenomas (Ried et al., 1996). However, in both tumours, recurrent and consistent specific chromosome aberrations occur at the transition from the high-grade adenomas/severe dysplasia to invasive tumour.

An increased level of gene expression occurs as a result of gene amplification and duplication involving whole or parts of chromosomes, which may affect the gene dosage of many genes simultaneously. Homogeneously stained regions and double-minutes are the common genetic structure of gene amplification (Kallioniemi et al., 1994). In primary breast cancer, 22% of all amplification found by CGH was in previously known amplified loci, and 78% was in other chromosome regions (Kallioniemi et al., 1994). This observation demonstrates the fact that studies limited to the currently known genes allow only a restricted view of the molecular mechanisms involved, and lead to an underestimation of the level of genetic instability in a particular cancer. The ability of CGH to detect amplification depends on the level of amplification and the size of the region affected. It is able to detect increases of a single copy of an entire chromosome, and 5-7 fold amplification of known oncogenes in homogeneous cell lines. CGH can also estimate the size of an amplified region. Distinctions between gene amplification and gain of whole chromosomes or chromosome arms are important. DNA gains range from high amplification of a small region (a few hundred Kb to 1-2 Mb) to entire chromosome arms. Primary tumours often show a copy number gain of large regions (up to tens of Mb), and this is likely to affect the expression of many different genes simultaneously. Simultaneous amplifications of DNA sequences originating from many different chromosome loci are common, and an amplicon may contain several highly expressed genes. Kallioniemi et al. (1994) suggested that genomic instability and the capacity for DNA amplification may be crucial factors in tumour progression, by upregulating numerous different genes. A genetically unstable cell would co-amplify random DNA sequences in association with an important oncogene, and this could have far reaching consequences for tumour development.
Appendix
Appendix A

(General standard solutions)

**PBS**

150 mM NaCl, 10mM Na$_2$HPO$_4$ / NaH$_2$PO$_4$, pH 7.4
1 tablet (Sigma) to 200ml sterile H$_2$O.

**RNAse**

Dessicated form was made into a stock solution of 10mg/ml in
2 x SSC, boiled 10 min and stored at -20°C.

**SSC**

15mM sodium citrate, 150 mM NaCl, pH 7, made up from a
20 x solution.

**TBE**

90 mM Tris-HCl pH 8.0, 90mM boric acid, 2mM EDTA.

**TE**

10 mM Tris-HCl pH 8.0, 0.1mM EDTA pH8 and autoclaved.

**TN**

0.1 mM Tris-HCl pH 7.5, 0.15 M NaCl.

**TNB**

TN + 0.5% blocking reagent (Boehringer), dissolved for 3
hours at 60°C, filtered twice through 1M filter paper
(Whatmann), stored at -20°C.

**TNE**

10 mM Tris-HCl pH 8.0, 0.2M NaCl, 1mM EDTA.

**TNT**

0.1 M Tris.HCl, 0.15M NaCl, pH7.5, 0.1% Tween 20 detergent
(Sigma).

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Appendix B
(Culture media and solutions)

**Colcemid**
10mls, 10 μg/ml in PBS without phenol (Gibco).

**Epidermal**
Human EGF (Sigma) stock solution 2 μg/ml used at 50μl/100 ml medium.

**FCS**
Fetal calf serum(Sigma)

**GPS**
100ml: 200mM L-glutamine(Gibco), 100U/ml penicilin and
100 μg streptomicin (Sigma), diluted to working concentration
of 1 % v/v and stored at -20°C.

**Hank's solution**
Stock solution 300ml: 32g NaCl, 1.6g KCl, 0.36g
Na₂HPO₄.12H₂O, 0.24g KH₂PO₄, 4g glucose, 0.08g phenol
red with ddH₂O, autoclave and stored at 4°C. Diluted 1:9 with
sterile H₂O and pH 7.0-7.2 with NaHCO₃ for working strength.

**Hydrocortisone**
5g anhydrous mol wt 262.5 cell culture reagent 98% (Sigma).

**Insulin**
250 μg/ml (Sigma).

**KCl EDTA**
3% KCl, 0.2% EDTA, 4.8% Hepes, pH 7.4 autoclaved and
hypotonic
solution

**KCl hypotonic**
0.075 M KCl
**DMEM Medium**  
9 mls x 10 Dulbecco's Modification of Eagle's medium (Gibco), 73 ml sterile deionised H₂O, 10 ml FCS, 2 ml GPS, 5 ml 5.3% NaHCO₃, 1 ml 1M NaOH.

**PHA**  
Phytohaemagglutinin (Gibco), 1 vial reconstituted in 10 ml H₂O, stored at 4°C.

**Versene**  
0.02g EDTA in 100 ml Hanks balanced salt solution as above and sterilised by autoclaving.

**Trypsin / Versene**  
5 ml 2.5% trypsin solution (Gibco) was added to 100ml versene before use and pH corrected with NaHCO₃/NaOH.

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**Appendix C**  
*(Banding solutions)*

**Buffer solution**  
Buffer tablets pH 6.8 (BDH, Gurr), 1 tablet in 1 litre dH₂O

**Bacto trypsin**  
1:250 trypsin, (Difco) 5%, stored at -20°C after rehydration with 10 ml sterile distilled water.

**Giemsa**  
BDH, Gurr

**SSC**  
as above
Appendix D
(Yeast media and solutions)

**Ampicillin**
200 μg / ml (stock solution 50mg / ml).

**S.D. medium**
7g / l Bacto yeast nitrogen base without aminoacids, 20g/l glucose, 55 mg/l adenine and tyrosine, autoclaved. Before use 56 ml/l 20% w/v filter sterilised casamino acids was added and stored at 4°C until use.

**S.D. agar**
10b/1 bacto-agar added to S.D. medium and autoclaved and stored at 4 °C until use.

**YLB**
100mM EDTA, 10mM Tris-HCl pH 7.5, 1% w/v lithium (Yeast lysis buffer) dodecyl sulphate.

**YRB**
1.2M sorbitol, 10mM Tris-HCl pH 7.5, 20mM EDTA, autoclaved. 14 mM β-mercaptoethanol was added immediately before use.

Appendix E
(Cosmid media and solutions)

**Antibiotic**
as for yeast cultures

**Cosmid broth**
100 ml: 1.2g tryptone, 2.4g yeast extract, 0.5ml glycerol, in deionised H₂O, 10 ml KPO₄ (12.5% K₂HPO₄).
**L broth**

1% tryptone, 0.5% NaCl, 0.5% yeast extract, in deionised H\textsubscript{2}O, autoclaved to sterilise.

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**Appendix F**

**(FISH solutions and buffers)**

**Anti-fade**

4 µl diamidino phenylindole (DAPI) 0.2 mg / ml and 4µl propidium iodide (1 mg / ml) were added to 1 ml of Vectashield mounting medium (Vector Labs) and stored at 4°C.

**Anti-avidin**

5µg/ml biotinylated anti-avidin D (Vector), was made up in 4 x SSC 0.5% Marvel, and stored at 4°C in the dark.

**Avidin**

5µg/ml fluorescein isothiocyanate (FITC) avidin DN (Vector) was made up in 4 x SSC/ 0.5% Marvel, and stored at 4°C in dark.

**Deionised**

100 ml: 5g ion-exchanger Amberlite monobed MB-1 mixed resin (BDH) and 100 ml formamide (Fluka/ BDH), were stirred in a sealed bottle in the dark for 2 hours, filtered twice through No 1 filter paper (Whatmann), and added immediately to the hybridization mix.

**Hybridization**

50% V/V deionised formamide, 10% W/V dextran sulphate in mix

2 x SSC were heated to 65°C to dissolve the dextran sulphate and this was stored at -20°C.
**Formaldehyde buffer**

50 mM MgCl₂ in 1 x PBS.

**Formaldehyde solution**

1.3 ml of 37% formaldehyde (Sigma) in 50 ml formaldehyde buffer

**PBS**

see Appendix A

**Proteinase K**

Stock solution 50μg/ml in proteinase buffer

**Proteinase K buffer**

20 mM Tris-HCl, 2 mM CaCl₂, pH 7.4

**10 x Phosphate buffer**

0.5 M NaH₂PO₄, Na₂HPO₄, pH 7.

**RNAse**

see Appendix A

**SSC**

" 

**Sorensen's buffer**

Concentrated buffer solution, diluted 1:10 to produce pH 6.8 (BDH)

**SSCM**

4 x SSC, 5% non-fat dry milk (Marvel), filtered through No 1 paper (Whatman).

**SSCT**

4 x SSC with 0.05% Tween 20 detergent (Sigma).
TN
see Appendix A

TNB
""'

TNE
""

TNT
""

Appendix G

Lysis buffer
100 ml: (1.21g) 100mM Tris.HCl pH 8.5, (0.19 g) 5 mM EDTA, (0.20 g) 0.2% SDS, (1.17g) 200 mM NaCl.
References


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Mitra AB, Rao PH, Pratap M (1994). i(5p) and del(6q) are nonrandom abnormalities in carcinoma cervix uteri. Cytogenetics of two newly developed cell lines. *Cancer Genet Cytogenet*, **76**:56-58.


Publications arising from this thesis


Award arose from this research

Molecular Cytogenetic Analysis of Five Newly Established Cervical Cancer Cell Lines Using G Banding and Fluorescence In Situ Hybridization

Angela T. A. Thein, Xin Han, Eadie Heyderman, Margaret Fox, Stuart J. Steele, and Jennifer M. Parrington

ABSTRACT: Cervical tumors nearly all have complex karyotypes and more precise cytogenetic information is required to establish whether specific rearrangements occur, and if they are related to the type of HPV infection found. The karyotypes of five recently established cervical cancer cell lines, three from squamous cell carcinomas (two HPV16+ve and one HPV 18+ve), one from an adenocarcinoma (HPV -ve), and one from an adenosquamous carcinoma (HPV 16+ve), have been analysed using fluorescence in situ hybridization (FISH), with 23 chromosome specific paints, YACs and cosmids as probes, in addition to conventional G banding, in order to identify markers and clarify the breakpoints. Chromosomes 1, 3 were rearranged in all cell lines. Breakpoints in the squamous lines were all in 3q, but in different regions. Small metacentrics involving chromosome 5 were a del(5q) in one line, and a t(X;5) in another, rather than i(5p). The region 6q21 was involved in three cases and chromosome 9 was rearranged in four. An i(8q) was found in three squamous carcinoma cell lines. Structural changes of 11q were found only in two cases, but a marker 11 representing amplification in the 11q14-2 region was duplicated in the adenosquamous line.

INTRODUCTION

There is a very limited amount of cytogenetic information available at present from direct preparations or short term cultures of invasive cervical cancer [1-4]. Reports published so far, have shown that tumors have a very varied modal chromosome number ranging from 40-100, and karyotypes with several unidentified markers. Chromosomes 1, 3, 11, 17 are involved most frequently in structural changes, and a small metacentric, an i(4p) or i(5p) is described as the most frequent single anomaly [2]. Conventional G banding of complex tumor karyotypes, such as these, nearly always results in a number of unidentifiable or misidentified regions and markers. Fluorescence in situ hybridization (FISH), using chromosome specific paint probes in conjunction with G banding, can be used to considerable advantage in this type of material [5]. Large numbers of good metaphase spreads are necessary for this technique. These are usually only obtainable from cultured cells, where abnormalities may be found which were not present in the original tumor. We have fully characterized the chromosome anomalies in five newly established cervical cancer cell lines with FISH painting, using twenty three different chromosome specific probes, in order to verify the accuracy of previous karyotyping with G banding. The increased efficiency of breakpoint detection should outweigh the disadvantages of using cell cultures. We show here that chromosomes 1 and 3 are always rearranged, that i(8q) and rearrangements of chromosome 6 and 9 are common, and that regions of amplification occur in some cell lines.

MATERIALS AND METHODS

Clinical details of the cervical tumors used in this study are shown in Table 1. Cell lines were established in long term monolayer culture using a mouse 3T3 feeder layer as described previously [6-8]. Cultures were maintained in DMEM supplemented with fetal calf serum, hydrocortisone, epidermal growth factor and insulin. DNA from the cell lines was used in DNA profile analysis, as before [6], to demonstrate that the lines were developed from different individuals without cross contamination. The human
Table 1 Clinical background of the cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage</th>
<th>Age</th>
<th>FIGO</th>
<th>Operation</th>
<th>Histology</th>
<th>HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM7</td>
<td>10-16</td>
<td>42</td>
<td>Ib</td>
<td>Wertheim's hysterectomy</td>
<td>poorly differentiated squamous cell carcinoma</td>
<td>18</td>
</tr>
<tr>
<td>EH2</td>
<td>8-12</td>
<td>57</td>
<td>Ila</td>
<td>Wertheim's hysterectomy</td>
<td>moderate to poorly differentiated focally keratinizing squamous cell carcinoma</td>
<td>16</td>
</tr>
<tr>
<td>DE3</td>
<td>14-25</td>
<td>40</td>
<td>Iib*</td>
<td>Pelvic exenteration</td>
<td>moderately differentiated keratinizing squamous cell carcinoma</td>
<td>16</td>
</tr>
<tr>
<td>IE6</td>
<td>9-14</td>
<td>62</td>
<td>III</td>
<td>Pelvic exenteration</td>
<td>moderate to poorly differentiated adenocarcinoma</td>
<td>—</td>
</tr>
<tr>
<td>XH1</td>
<td>6-44</td>
<td>32</td>
<td>Ib</td>
<td>Wertheim’s hysterectomy</td>
<td>moderately differentiated focally keratinizing adeno-squamous carcinoma</td>
<td>16</td>
</tr>
</tbody>
</table>

*The tumor sample from which the DE3 cell line was derived was taken from the second recurrence, six years after the original tumor (FIGO stage IIb) was diagnosed. Radiotherapy was given at the time of first diagnosis and radical surgery was performed at the time of first recurrence.

Papilloma virus (HPV) infection status was determined by the polymerase chain reaction (PCR) as described previously [6]. Metaphase spreads for G banding and FISH were prepared using standard methods. Best results were obtained if cells were trypsinised two to three days prior to harvest, and medium changed 24 hours before to remove any floating cells.

A total of 23 biotin labelled human chromosome specific paint probes (1-22 & X) (CAMBIO), supplied with the appropriate amount of competitor DNA were used in this study. The three cosmid probes used were as follows: D3S939 (3q27) and D3S673 (3p24) from the Japanese Cancer Research Bank (ICRB)-gene, National Institute of Health, Tokyo, and 256C3 (9q34) from the Lawrence Livermore National Laboratory chromosome 9 specific library. The four YAC probes all from the HGMP resource centre, were as follows; 262 (3p21), 36(3p25-26), 303 (3q21) and 168 (3q24-26). The Fluorescence in situ Hybridization Chromosome painting procedure was based on the protocol by Pinkel et al [9] which we have described previously [5]. The cosmid and YAC DNA probes were labelled with biotin or digoxigenin by nick translation according to the

Figure 1 Representative Karyotype of SM7. [the del(10p) was observed in only 2 out of 10 cells]. See Table 2 for complete karyotype description.
Molecular Cytogenetics of New Cervical Cancer Cell Lines

Table 2

<table>
<thead>
<tr>
<th>Cells lines</th>
<th>Modal no.</th>
<th>Range</th>
<th>Karyotypes</th>
<th>Fig. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM7</td>
<td>91</td>
<td>88-96</td>
<td>91</td>
<td>31</td>
</tr>
<tr>
<td>EH2</td>
<td>88</td>
<td>87-91</td>
<td>88</td>
<td>31</td>
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<tr>
<td>DE3</td>
<td>77</td>
<td>74-80</td>
<td>77</td>
<td>31</td>
</tr>
<tr>
<td>JE6</td>
<td>52</td>
<td>74-86</td>
<td>52</td>
<td>31</td>
</tr>
<tr>
<td>XHl</td>
<td>78</td>
<td>75-86</td>
<td>78</td>
<td>31</td>
</tr>
</tbody>
</table>

method described by Rigby et al [10]. Prehybridization treatment included RNase A and proteinase K. Denaturation and posthybridization procedures were as described previously [5].

Images were viewed either on a Nikon Optiphot microscope and captured, using an MRC 600 (BioRad) confocal laser microscope attachment, (Figs. a, c, d & e), or on a Zeiss Axiophot with a cooled CCD camera and Smart Capture system (Digital Scientific, Cambridge) (Figs. b & f).

RESULTS

Cell Cultures

With the exception of DE3 (Table 1), all cell lines were derived from the original tumors when they were first diagnosed. All except JE6 were HPV positive, three for type 16 and one for type 18 (Table 1). The DNA profile analysis using the hypervariable minisatellite probe, MS31, demonstrated that each line has its own characteristic DNA pattern, which was different from that of the other lines, confirming that they had been developed from different individuals, and that there was no cross contamination between them.

Modal Chromosome Numbers

Modal numbers for the three squamous cell lines are shown in Table 2. With the exception of JE6, all modal numbers were in the triploid / hypotetraploid range.

Karyotypes

Karyotype analysis using G banding and FISH was carried out over several passages as shown in Table 1. On the whole, the karyotypes remained quite stable, and there was not a great deal of variation between different cells. The exception was the adenoid-squamous line, XH1, which when first examined at passage 6[6], had several markers which did not appear in later passages. Chromatid breaks were seen in several cells at this stage. Unfortunately, it was not possible to examine early passage cells with FISH, so these early markers remain unidentified. Nevertheless,
at least seven of the markers present at early passages could be identified later with FISH, and are shown in the composite karyotype (Fig. 2). G band karyotypes and the position of breakpoints modified according to the FISH painting results, are shown in Figures 2-5. Examples of rearrangements revealed by FISH are shown in Figures 6-9. Chromosomes involved in rearrangements and all independent breakpoints are shown in Table 2. It can be seen that chromosomes 1 and 3 are involved in all five cell lines.

**Chromosome 1**

Ten independent breakpoints were seen altogether in chromosome 1, including 2 at 1q21 and two at 1q10. Isochromosomes were seen for both 1p and 1q. Rearrangements occurred between 1p and three different chromosome regions; 3p14, 6q21, 20p11, in XH1, and between 1q and two different regions; 11q13 in EH2, and 20p11 in DE3.

**Chromosome 3**

An i(3q) was found in two lines, and chromosome 3 was also involved in different rearrangements with chromosomes 1, 2, 4 and 5. In all three squamous lines, breakpoints were on 3q. Breaks on 3p were only found in the adenocarcinoma and adeno-squamous lines. YACs and cosmids were used to locate the breakpoints more precisely.

In SM7, (Fig. 2b), the centromeric alpha satellite 2 probe was used to identify the marker, der(2)t(2;3). Both the digoxigenin labelled cosmid from 3p24 (red signals), and the biotin labelled cosmid from 3q27 (green signals), were observed on the three normal copies of chromosome 3, but only the biotin labelled cosmid from 3q27 was seen on both arms of the isochromosome 3. On the der(2) marker, the green cosmid signals from 3q27 were present, so the breakpoint was likely to be at 3q26.

In EH2, signals from the 3q21 YAC were only observed on the three normal copies of chromosome 3 and not on the der(4)t(3;4) marker. Therefore the breakpoint must be lower than 3q21. Signals from the 3q24 YAC were seen on the marker as well as on the normal chromosomes 3, so the breakpoint was assigned to 3q22.

In DE3, 3p25 YAC signals, were seen on the normal chromosome 3 and on the der(5) translocation product (Fig. 2d), but were absent from the der(3), showing that the breakpoint was below 3p25. In XH1, (Fig. 2f), the green signal, from paint 1 is seen on the der(3)t(1;3). The digoxigenin labelled 3p21 YAC was found on the normal chromosome 3, but no red signals were present on the der(3), so the breakpoint was proximal to the probe and put at 3p14.

**Other Chromosomes**

All the cell lines from squamous cell carcinomas had an isochromosome for the long arm of 8, i(8q), and also break-
points in 9 and X. Chromosome 9 was rearranged in four, and chromosomes 2, 4, 5, 6, 8, 12, 14, 15 and X in three out of the five cell lines.

Common Breakpoints
There were no breakpoints which were common to all five cell lines, but several which occurred in more than one. Apart from breakpoints 1q10, 3q10 and 8q10 involved in isochromosomes, they included 1q21, 6q11, 6q21, 14p11, 15p11, 20p11 and 21p11. The 6q21 region was involved in three out of five cell lines.

Sites of Amplification
Fish painting also revealed two regions of amplification double minutes in early passage SM7 cells which showed signals with chromosome 1 paint, and an extension of the 11q14-22 region in XH1.

DISCUSSION
Although a specific chromosome marker has yet to be found in association with cervical cancer, a high frequency of chromosome 1 rearrangements has been shown, consistently, in direct preparations from cervical tumors [2–4]. In the present investigation of cervical tumor cell lines the highest number of breakpoints (ten), occurred in chromosome 1. Double minutes, found initially in one squamous line (SM7), were also found to originate from chromosome 1. Genes on this chromosome are thought to have an important role in cell immortalisation [11], and may be disrupted by integration of viral sequences from human papilloma viruses (HPV) [12], or herpes simplex (HSV) [13], both of which have been associated with cervical carcinoma. Epithelial cells immortalized in vitro with HPV show alterations of chromosome 1 before they become tumorigenic, suggesting that these changes could be early genetic events in cervical cancer [12]. Deletion of tumor suppressor genes and activation of oncogenes could follow as the tumor develops.

Likewise, chromosome 3 was rearranged in all five cell lines, but extensive investigations with YACs and cosmids showed that no consistent breakpoint occurred, although isochromosomes for the long arm, and breaks in the short arm, could have resulted in loss of heterozygosity for loci on 3p, which has been reported in up to 70% of cervical cancers by several groups [14–18]. Rearrangements of chromosome 3 are common in HPV immortalized human cell lines [19], but it may be necessary for specific translocation involving oncogenes such as ETS 2 (on 21q22), to occur, before cells become tumorigenic [20]. Popescu and co-workers [21] have shown that cervical cells, transformed in vitro with HPV, only had chromosome 3 abnormalities when they became tumorigenic, after transfection.
with another oncogene H-ras. On the other hand, rearrangements of chromosome 3 are also found in HPV negative cervical carcinoma cell lines such as HT-3 [22], and, as shown here, in JE6, the only HPV -ve line in our series, which has a very stable hyperdiploid karyotype with only six rearrangements including a 3/5 translocation. Breaks in 3q have also been described in two newly developed cervical cancer cell lines in which the HPV status is not defined [23]. Clearly, disturbances of genetic material in chromosome 3 are an important factor in cervical cancer, whether or not the HPV genome is involved.

A small metacentric chromosome thought to be an i(5p), has been found at quite a high frequency in some investigations [2, 23 and 24] but not in others [3]. Small metacentrics involving chromosome 5 were seen in our cell lines, SM7 and XH1, but they were different from each other; an X/5 translocation and a 5q deletion respectively. It is possible that an i(5p) could have been lost during cell cultivation, but specific isochromosomes like the i(12p) found in testicular tumors, are usually retained [25].

A del(6q) very similar to the one described by Mitra et al [23] in two recently developed cervical cancer cell lines was seen in one of our cell lines (SM7), but in this case the breakpoint was nearer the centromere (6q11). Another breakpoint, however, was found in three of our cell lines (SM7, EH2 & XH1), further down the long arm, at 6q21, within the common region of deletion described by Mitra [23]. A recent LOH study which investigated loci on all autosomal arms in cervical cancer [18], has shown that the frequency of LOH on 6p (43%) was even higher than 3p (39%). LOH on 6q was lower, but still above 20%, so tumor suppressor genes on this chromosome may also be important in the development of cervical cancer.

The i(8q) found in the three squamous carcinoma cell lines, all of which carried HPV DNA, is interesting in view of the common HPV integration site found by others at 8q24, close to the myc oncogene [12]. The exact integration site in our cell lines has yet to be established, but the 8q:12q translocation in SM7 is very similar to the one described by Gallego et al [26], in which they demonstrate HPV 18 integration at 8q22.1.

It is interesting to note that chromosome 9 abnormalities were seen in four out of five of our cell lines, two with breakpoints in 9p and four in 9q. This chromosome does not show high levels of LOH in cervical cancer [16 and 18], but the breakpoints in our cell lines were in the region of the multiple tumor suppressor gene (MTS-1) at 9p21-22 [27], more proximal to those described by Zimonjic at 9p24 in two HPV negative cervical carcinoma cell lines, where extensive rearrangement of chromosome 9 was seen [22].
Although LOH is quite frequent on chromosome 11 [18] and the Hela tumor suppressor gene is thought to be located on this chromosome [27] and 11q structural changes have been seen frequently in direct preparations [2], we only found 11q breakpoints in two of the cell lines (EH2 and XH1). However, in XH1, the marker 11 was composed entirely of chromosome 11 material suggesting amplification of DNA in the 11q14-22 region.

Breakpoints in tumor cell lines can be described much more precisely after FISH, especially if YAC and cosmids probes are used as well as chromosome specific paints. After carrying out just such a FISH analysis on five cervical cancer cell lines, we have shown that chromosomes 1, 3, 6 and 9 are the most frequently rearranged, and could be the sites of oncogene activation and/or tumor suppression involved in the development of this type of tumor.

ATAT was supported financially throughout this project by a grant from the Newman Foundation. The MCR 600 confocal laser microscope attachment was provided by a grant from the MRC UK Human Gene Mapping Project. YACs and cosmids were obtained via Professor MS Povey, Dr. JD Delhanty, Dr. K Hashimoto and Dr. Y Nakamura. We would also like to thank Dr. D Griffin for useful advice on FISH procedure.

REFERENCES


Molecular Cytogenetics of New Cervical Cancer Cell Lines


Figure 6 FISH using chromosome specific paint probes.
(a) Paint 3 onto a metaphase from SM7 showing four normal copies of chromosome 3 and the t(2;3)(arrowed).
(b) Partial metaphase from SM7 showing cohybridisation of the alpha-satellite chromosome 2 probe (larger green signals), with probes from 3q27 (smaller green signals) and 3p24 (red signals). The region 3q27 is found on the der(2) (large arrow-head). The three normal copies of chromosome 3 have green signals at one end and red signals at the other (medium arrow-heads). The isochromosome 3 has green signals at both ends (smallest arrow).
(c) Paint 3 onto a metaphase from JE6 showed one normal copy of chromosome 3 and both parts of the reciprocal translocation, t(3;5)(p24;q31) (arrowed).
(d) Metaphase from JE6 showing 3p25 YAC signals on the normal chromosome 3 (small arrow), and on the der(5), (large arrow), but not on the der(3), (arrow-head).
(e) Paint 3 onto a metaphase from XH1 showing three normal copies of chromosome 3 and the der(3)(1;3), (arrowed).
(f) Partial metaphase from XH1 cohybridized with paint 1 (green signal), and the 3p21 YAC (red signal), showing the der(3) (arrowed) with chromosome 1 paint but no red signal (3p21 region).