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Analysis of chromosomes 3, 7, X and the EGFR gene in uterine cervical cancer progression

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Abstract

The aim of this study was to investigate the possible role of genetic alterations in the genesis and progression of cervical carcinomas. We analysed the 3, 7, X aneusomy of chromosomes and the status of the epidermal growth factor receptor (EGFR) gene by fluorescence *in situ* hybridisation (FISH) analysis. Polysomy of chromosomes 3 and X defined the transition from high-grade squamous intraepithelium lesions (HSIL) to cervical carcinoma. Chromosome 7 monosomy and polysomy did not show any statistical significant differences between the groups examined. When we compared the chromosomal aneusomies in all of the specimens using the Kruskal–Wallis test, significant differences ($P = 0.0001$, $P = 0.0001$ for chromosomes 3 and X, respectively) were observed. Using a ratio of the EGFR gene signals and chromosome 7 centromeric signals, no samples showed gene amplification. Our results demonstrate the importance of chromosomal 3 and X aneusomies in the development and progression from HSIL to cervical carcinoma, highlighting their usefulness as genetic markers for identifying SILs at high-risk of progression.

Keywords: Genetic markers; Cervical carcinoma; Progression

1. Introduction

The development of cervical carcinoma is preceded by distinct morphological changes from normal epithelium to carcinoma through low-grade and high-grade Squamous Intraepithelium Lesions (LSIL and HSIL). The genetic basis of this progression is poorly understood [1,2]. Infection with the human papillomavirus (HPV) is considered to be the major aetiological factor in cervical carcinogenesis. However, most HPV-induced cervical lesions are reversible [3]. In fact, oncogenic types of HPVs have been found in more than 95% of cervical carcinomas, but only a small fraction of women harbouring oncogenic HPV in their lower genital tract will develop cervical cancer. Thus, HPV infection alone appears to be insufficient for progression to a malignant phenotype and suggests the involvement of other genetic and/or epigenetic events in cervical carcinogenesis. Such events may be chromosomal aberrations affecting tumour suppressor genes or cellular oncogenes. According to the multistep nature of carcinogenesis, cervical lesions should have a sequential accumulation of genetic changes as they progress towards malignancy and invasion. The identification of recurrent chromosomal aberrations is important in order to understand cervical carcinogenesis. Recurrent patterns of chromosomal alterations with specific imbalances that are important for tumour initiation and progression have been observed in cervical cancer [4]. A number of studies have shown

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frequent loss or gain of several chromosomes in cervical cancer, such as chromosomes 3, 8, 5, 7, X and 18 [5,6]. Cellular proto-oncogenes play a central role in cell growth regulation. Studies in different types of cancer have shown that increased expression of the epidermal growth factor receptor (EGFR) gene is generally associated with a more aggressive biological behaviour compared with tumours with low or normal expression [7]. It has been reported that overexpression of EGFR in cervical carcinoma correlates with a poor prognosis [8]. In this study, to investigate the possible role of genetic alterations in the genesis and progression of cervical dysplasia, 67 cervical lesions of varying histological grades were examined by fluorescence *in situ* hybridisation (FISH) for chromosomes 3, 7 and X and EGFR gene alterations.

2. Materials and methods

2.1. Clinical samples

The characteristics of the patients and histological classifications are given in Table 1.

Eighty-one women, average age 46.14 years (ranging from 22 to 75 years), were included in this study. All the patients underwent a pelvic examination, Papanicolaou (Pap) Test and colposcopy plus biopsy.

Cytological, according to the Bethesda System [9], and histological diagnoses were performed and revealed LSIL in 19 cases, HSIL in 28 cases and squamous invasive cervical cancer in 20 cases. Tumour grade was available for the 20 tumours and included 1 low-grade (grade 1), 9 moderate-grade (grade 2) and 10 high-grade (grade 3) tumours. Twenty samples of benign lesions of uterus (fibromatosis) were used as controls as a measure of the inherent probe hybridisation efficiency.

Table 1
Patient Characteristics

Parameters	*N*
Age (years)	81
Mean (range)	46.14 (22–75)
Histological type	
Fibromatosis	14
LSIL	19
HSIL	28
*Cervical carcinoma	20
*FIGO stage	
IA_1–B_2	6
II B	8
III B	6

LSIL, Low-grade Squamous Intraepithelium Lesions; HSIL, High-grade Squamous Intraepithelium Lesions; FIGO, International Federation of Gynaecology and Obstertrics.

All of the slides were reviewed by two different gynaecological pathologists.

Patients with LSIL underwent conservative methods of treatment with laser destruction. Patients with HSIL underwent conservative methods of treatment, either with a electrodiathermy loop (LLETZ), if the patient was nulliparous with a small lesion, or cold-knife conisation for large lesions.

Patients with invasive cervical cancer (stage International Federation of Gynaecology and Obstetrics (FIGO): IA_1-IIIB) underwent either hysterectomy or chemoradiotherapy. All classifications were made without prior knowledge of the cytogenetic findings.

2.2. FISH analysis

All samples were immediately used for the FISH evaluation. Cytogenetic studies were done using cytological imprints of the surgical biopsy. Touch preparations (imprints) were fixed immediately after collection with Carnoy's fixative. The use of touch preparations was chosen to circumvent difficulties in interpretation attributable to sectioned nuclei and lower hybridisation efficiencies in standard pathological specimens. The touch preparations were reviewed by a pathologist to verify the presence of adequate cellular components.

2.3. Probes

The chromosome enumeration probes (CEP) were pericentromerically fluorescent-labelled for use in the FISH assay (Vysis, Inc., Downers Grove, IL) and were specific for the centromeric region of chromosome 3 (3p11.1–q11.1), 7 (D7Z1) and X (DXZ1). The LSI (Locus Specific Identifier) EGFR probe contains DNA specific sequences for the EGFR human gene locus and hybridises to the 7p12 region of human chromosome 7. The CEP 7 was used as a control to determine the copy number of chromosome 7 when the EGFR copy numbers were counted. Ratios of the average copy numbers per cell were calculated to establish the presence of amplified EGFR. Target DNA on the slides and the probe mixtures were denatured together (2′ at 72 °C) and hybridised using the Hybrite system (Vysis, Inc.). After 4 h of hybridisation at 37 °C, a post-hybridisation wash was applied (0.4 × SSC/NP40 at 73 °C). The slides were then counterstained using DAPI (4,6-diamino-2-phenylindole) and processed with an Olympus BX60 fluorescence microscope equipped with a 100 watt mercury lamp. Separate band pass filters were used for the detection of the CEP 3 and EGFR probe signals (spectrum orange) and for the detection of the CEP 7 and X (spectrum green) probe signals. After DAPI counterstaining, fluorochrome signals were captured individually and images were generated via a computer using the Quips Genetic Workstation and Imaging

Software (Vysis). The amplification of the EGFR gene was defined as a EGFR to CEP 7 ratio greater than 2.

2.4. Statistical analysis

Our statistical evaluation was based on the median value of monosomic and polysomic cells. Mann–Whitney and Kruskal–Wallis tests were used for the statistical analyses and P values of < 0.05 were considered statistically significant. We used the Pearson's test when we compared the chromosomal aneusomies with the patient's age.

3. Results

All 20 patients in the control group with cytological results within morphological normal limits showed a regular diploid chromosomal pattern for the chromosomes evaluated (data not shown). Results of our genetic evaluation are given in Table 2. Polysomy of chromosomes 3 and X were the most consistent chromosomal aberrations in cervical carcinoma. In addition, they defined the transition from HSIL to cervical carcinoma: in fact, as illustrated in Table 3, statistical differences were observed ($P = 0.0001$ and $P = 0.0001$, respectively) when we compared the chromosomal 3 and X aberrations in the HSIL and cervical carcinoma groups. As far as chromosomal 7 aberrations are concerned, no significant differences emerged between the groups examined. No statistical differences emerged between the LSIL and HSIL groups for chromosomes 3, 7 and X. When we compared the chromosomal polysomy in all three groups examined by the Kruskal–Wallis test, significant differences ($P < 0.0001$ and $P < 0.0001$ for chromosomes 3 and X) were present. In fact, the polysomy, with the progression from LSIL to cervical carcinoma, increases 3 and 6.4 times for chromosomes 3 and X, respectively. Regarding monosomy, only chromosome 3 showed significant differences ($P = 0.039$) when we compared the HSIL and cervical carcinoma groups (Table 3). Since eight samples (1 HSIL and 7 cervical carcinoma) showed chromosome 7 polysomy higher than 20% (Fig. 1), we evaluated the status of the EGFR gene using CEP 7 to adjust for the effects of aneuploidy and to establish the presence of true amplification. Our results always showed ratios (EGFR:CEP7) lower than 2 (Fig. 2). No samples showed gene amplification. Mean losses and polysomies did not show a positive correlation with the patient's age. Even if there was no statistically significant relationship, a positive trend was present in the LSIL group

Table 2
Median values and ranges of chromosomal aberrations (monosomy and polysomy) in each sampling group

	N°	Chromosome 3		Chromosome 7		Chromosome X	
		Monosomy	Polysomy	Monosomy	Polysomy	Monosomy	Polysomy
LSIL	19	9(2–22)	15.5(2–56)	12(6–40)	3(0–18)	11(2–42)	5.5(2–42)
HSIL	28	13(4–32)	9(4–64)	14(0–30)	2(0–32)	12(0–40)	17(0–56)
Cervical carcinoma	20	6(0–22)	47(10–100)	13(0–32)	6(0–96)	7.5(2–32)	35(0–96)
Total	67						

CC, cervical carcinoma.

Table 3
Statistical analysis after FISH evaluation for chromosomes 3, 7, X

		P value			
		LSIL vs. HSIL[a]	LSIL vs. tumour[a]	HSIL vs. tumour[a]	LSIL vs. HSIL vs. tumour[b]
Chromosome					
3	Monosomy	NS	NS	0.039	NS
	Polysomy	NS	0.008	0.0001	0.0001
7	Monosomy	NS	NS	NS	NS
	Polysomy	NS	NS	NS	NS
X	Monosomy	NS	NS	NS	NS
	Polysomy	NS	0.0001	0.0001	0.0001

NS, non-significant.
FISH, fluorescence *in situ* hybridisation.
[a] Mann–Whitney test (non-parametric test).
[b] Kruskal–Wallis test.

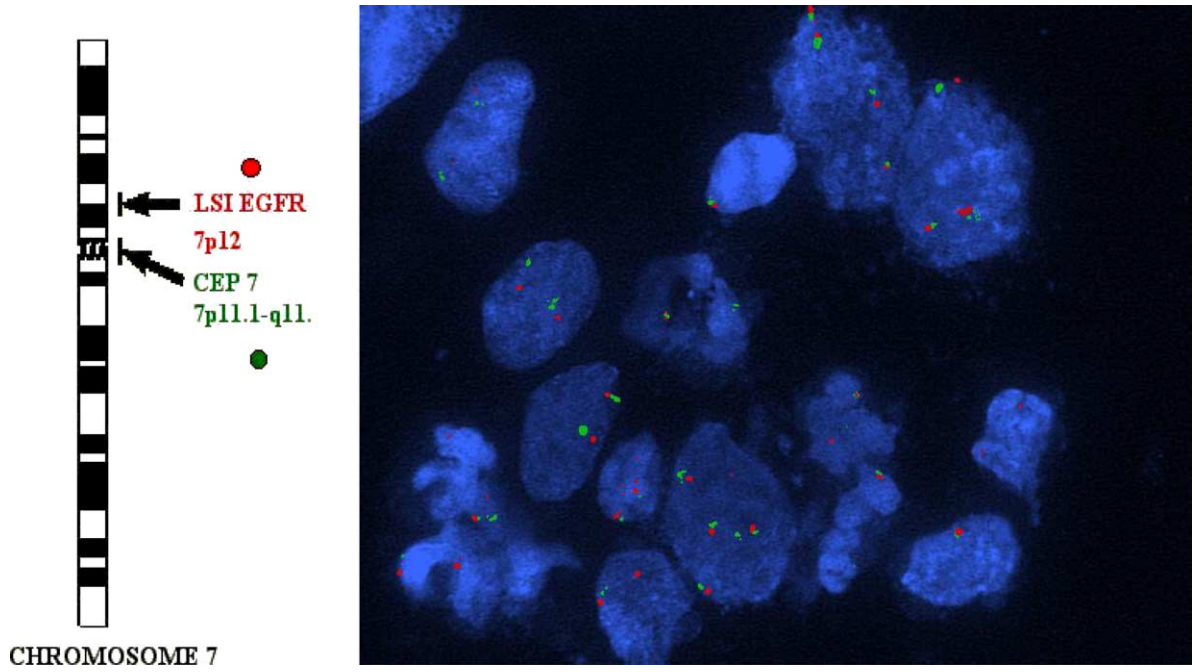

Fig. 1. Representative photomicrograph of non-amplified (EGFR) gene in cervical cancer cells after fluorescence *in situ* hybridisation (FISH) with EGFR (orange) and chromosome 7 (green) specific probes. Ratio CEP 7/EGFR<2. Magnification is 100×.

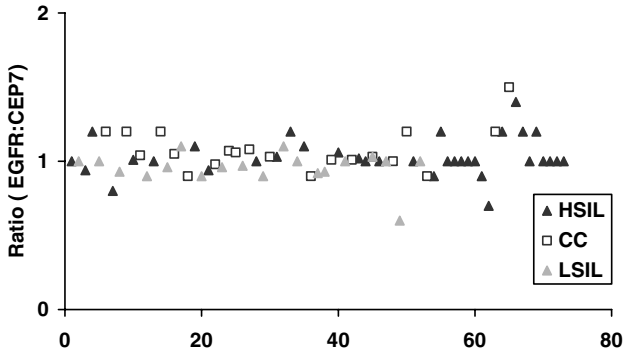

Fig. 2. Distribution of ratios (EGFR:CEP 7) in cervical carcinoma (CC), HSIL and LSIL specimens.

for all of the chromosomal aneusomies analysed, but only for chromosome X aneusomy in the cervical carcinoma group.

4. Discussion

Converging points of evidence implicate infection by high-risk HPV types as a critical aetiological factor in cervical tumorigenesis. However, epidemiological and experimental data show that only a small fraction of HPV-infected squamous intraepithelial lesions progress to invasive cervical carcinoma [8]. However, the positive predictive value of HPV testing is limited, especially in young sexually active women, among whom transient innocuous infections are very common [10]. These findings suggest somatic genetic mutations play a critical role in the initiation and progression of cervical carcinoma. Delineation of these genetic changes is crucial to obtain an understanding of the molecular basis of cervical carcinoma. Premalignant lesions of the uterine cervix represent a pathological continuum of mild to severe epithelial dysplasias. In this work, we analysed a number of preinvasive cervical lesions, as well as invasive cervical carcinomas, in order to identify characteristic chromosomal changes related to different stages of cervical tumour progression. Genetic alterations are often described as influencing tumour behaviour [11,12] and may therefore serve as useful prognostic factors. Heselmeyer and colleagues [13] proposed that a gain on chromosome arm 3q is an event in cervical carcinogenesis that occurs during the transition from premalignant lesions to invasive carcinoma. Furthermore, they suggested [14] that gains of chromosomes 1q, 3q and 5p could be particularly frequent in advanced-stage cervical carcinomas (clinical stage IIB-IV). Mian and colleagues [5] confirmed that trisomy 7 may be considered an early event in cervical carcinoma, persisting and increasing with the progression of the lesion. In this genetic evaluation, corresponding patterns of characteristic aberrations were detected in both intraepithelial lesions and invasive tumours, although the number of aberrations was much higher in the latter category. Our results demonstrate a close genetic relationship between all of the examined tumours, HSIL and LSIL groups. For chromosomes 3 and X, our cytogenetic findings re-

vealed an accumulation of chromosomal aberrations during neoplastic transformation, while the frequency of aneusomy of chromosome 7 was similar in all three groups (Table 3). Particular attention should be paid to the chromosomal 3 and X aberration that were observed. Chromosome 3 and X polysomy were not only the most consistent aberrations found in cervical carcinomas, but also defined the transition from HSIL to cervical carcinoma. This chromosomal instability was confirmed by the high levels of monosomy observed in all of the groups examined. We propose that chromosomal 3 and X alterations in premalignant lesions could be considered a potentially useful intermediate biomarker of tumorigenesis to detect patients at high risk of cervical carcinoma who may benefit from preventive intervention(s). The interpretation of the hybridisation patterns provides evidence that chromosomal 3 and X instability, measured in this study as the variability of hybridisation patterns among cells in a given case, increases with the grade of dysplasia as well. The presence of monosomy and polysomy suggests that early chromosomal aneuploidies can develop in an morphologically normal nucleus. Our data for chromosome 7, disagrees with the results of Bulten and colleagues [15], who demonstrated that aneusomy of chromosome 7 seems to be an early event in the oncogenesis of cervical carcinoma, but is not a marker for the progression of premalignant lesions. There is emerging data regarding the use of EGFR amplification as a prognostic marker and/or therapeutic target [16] in cervical cancer. Kersemaekers and colleagues [8] reported that overexpression of the EGFR in the uterine cervix is associated with a poor prognosis. The EGFR gene copy number could be increased either due to specific gene amplification or by increasing the copy of its corresponding chromosome, and could affect protein expression due to a gene dosage effect. Therefore, we evaluated the status of the EGFR gene as a ratio with the centromeric signals of chromosome 7. The results showed no amplification in the three groups examined. Conflicting results in the literature may be related to the different methodologies used to assess EGFR gene status and/or could indicate that mechanisms, other than amplification, could also result in high EGFR expression [17]. Our data highlight: (i) the importance of increased DNA copy number of chromosomes 3 and X in the development and progression from HSIL to cervical carcinoma; (ii) that the status of chromosomes 3 and X could be a useful genetic marker for identifying squamous intraepithelial lesions at high-risk of progression; (iii) alterations of chromosome 7 seems to be an early event in oncogenesis.

Recently, the development of assays with high-specificity for detecting cancer precursors, as well as with excellent sensitivity, represent attractive alternatives as primary screening tests or as tests to complement cytology, HPV-typing or other assays. Furthermore, we conclude that the FISH assay, with probes specific for the chromosomes involved in cervical carcinogenesis, is a valuable tool to identify women with LSIL who harbour undetected HSIL or are destined to progress and may help determine their risk of progression.

5. Conflict of interest statement

No authors has financial and personal relationships with people or organisations that could inappropriately influence our work.

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