Research Article

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Biological characteristics of cervical precancerous cell proliferation

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Abstract: High-grade squamous intraepithelial lesions (HSILs) are regarded as precancerous lesions that can progress to cervical carcinoma; however, it is very difficult to effectively differentiate these precancerous cells from cancerous cells based on morphology alone. Additionally, the difference between precancerous cells and cancerous cells in regard to biological behaviour remains unclear. We previously cultured primary normal uterine cervical keratinocytes from human normal cervical tissue and cervical precancerous cells that were naturally infected with human papillomavirus from small-sized neoplastic cervical tissues. Here, we extended our study to further observe the in vitro proliferative characteristics of cervical precancerous cells at the cellular and molecular levels. In this study, we found that the growth rate of precancerous cells was significantly faster than that of normal cervical cells and slower than that of Caski cells. However, the proliferative capacity of such precancerous cells was similar to that of cancerous cells of the cervix at the molecular level. These results suggest that the surrounding environment of the cells may play an important role in the development of cervical cancer, which provides an important basis for the further study of precancerous and cancerous lesions of the cervix.

Keywords: Biological behaviour, cervix, proliferation, precancerous conditions

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1 Introduction

Cervical cancer is the most common gynaecological cancer in developing countries and is preceded by a long phase of squamous intraepithelial lesion (SIL), which is classified based on histology according to the severity [1-3]. High-grade SIL (HSIL) persists and progresses to cervical cancer in approximately 20% - 45% of untreated lesions and is regarded as a precancerous lesion of the cervix [4, 5]. HSIL, also known as high-grade cervical intraepithelial neoplasia (CIN), is characterized by four major pathological microscopic changes, including the presence of cells of unequal size, abnormally shaped cells, hyperchromatism and the presence of mitotic figures [4, 6]. However, it is very difficult to differentiate dysplastic cells from cancerous cells to any extent based on morphology alone. Additionally, carcinoma in situ, which is considered a special type of HSIL, is distinguished from cervical carcinoma only because of its intact basement membrane; the mean interval of progression in cervical cancer precursor lesions that progress to invasive cancer is approximately 10-20 years [4, 7]. Furthermore, early-stage cancerous cervical lesions that have invaded the stroma are surrounded by precancerous lesions. Therefore, it is important to determine the difference between such precancerous cells and cancerous cells in regard to their biological behaviour. To date, no studies have been published regarding the proliferative characteristics of precancerous cells of the cervix in vitro. This is due to the technical and methodological problems associated with the in vitro culture of these cells [8], despite the large amount of clinical research that has been performed on precancerous and cancerous lesions of the cervix [4, 5].

It is generally accepted that cell proliferation ability may be evaluated at both the cellular and molecular levels. Cell growth curves are regarded as a common and important parameter that may be used to assess cell proliferation at the cellular level. Some authors have reported the cell growth characteristics of primary cultured cervical carcinoma cells and have noted that the curves of uterine cervical cancer cells were of the 'S' type with a 24-48 h adaptive phase and a cell population doubling time of

26-30 h [9, 10]. However, no study has been performed to investigate the cell growth characteristics of cervical precancerous cells [11]. Cell cycle analysis is widely used to assess cell proliferation capacity at the molecular level. Additionally, both the S-phase fraction (SPF) and DNA aneuploidy have been demonstrated to be biomarkers of tumour development because of their relationship to the cell cycle state [11]. Indeed, DNA aneuploidy results from the genetic and chromosomal instability of cancer cells and has been reported as a prognostic and predictive biomarker of cancer [12, 13]; moreover, the S-phase fraction (SPF) roughly corresponds to the fraction of cells that are proliferating. Data from the literature indicate that both the SPF and DNA aneuploidy have been extensively investigated, but the results have been contradictory and are confined to clinical studies [11, 14, 15]. Until now, very little was known about the proliferative capacity of precancerous cells.

Accordingly, we speculated that some of the biological behaviours of the precancerous cells are similar to those of cancerous cells, and the surrounding environment of the cells may play an important role in the development of cervical cancer. In our previous study, we established a simple and practical method to rapidly obtain highly purified normal uterine cervix (NUC) keratinocytes from human normal cervical tissue and to obtain cervical precancerous cells that were infected naturally with human papillomavirus (HPV) from small-sized neoplastic cervical tissues [16, 17]. Based on these grounds, we aimed to evaluate the proliferative capacity of cervical precancerous cells in vitro at the cellular and molecular levels and to compare their proliferative capacity with that of NUC and Caski cells, therefore providing an experimental basis and evidence for the further study of precancerous and cancerous lesions of the cervix.

2 Materials and methods

2.1 Cell culture

The culture of cervical precancerous cells and NUC keratinocytes was conducted as described in our previous study [16, 17]. Briefly, small pieces of tissue (2-4 mm^3) that were collected from patients with naturally HPV-infected cervical HSILs, with the informed written consent of the patients, were digested with 0.2% type I collagenase (Gibco, Grand Island, NY, USA) solution in a gas bath thermostatic oscillator at 37°C and 200 r/min for 40 min. The isolated cells were seeded into T25 plastic tissue culture flasks that were pre-coated overnight with 2 μg/cm^2 collagen type I from the rat tail (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured at room temperature in keratinocyte serum-free medium (K-SFM) (Gibco, Grand Island, NY, USA). The medium was supplemented with 5% foetal bovine serum (FBS) (HyClone, Logan, UT, USA) and antibiotics (2.5 μg/ml amphotericin B, 100 IU/ ml penicillin, and 100 mg/ml streptomycin) at a cell density of 7-10 × 10^4 cells/cm^2. The cells were cultured in a CO_2 incubator at 37°C and were used in experiments at passages 2-4. The medium was changed twice per week. The identification of such cervical HSIL cells was conducted as described in our previous study [17].

Small tissue fragments from NUC that surrounded the cervical precancerous lesion were used as negative controls. The cervical cancer cell line Caski was purchased from the American Type Culture Collection (Manassas, VA, USA); these cells were cultured in RPMI-1640 medium supplemented with 10% FBS and served as the positive control.

2.2 Cell growth curves

The growth curves of cervical precancerous cells and the controls were measured by an MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The cells were treated with 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) after they entered the logarithmic growth phase and reached a growth plateau. The cell density was adjusted to 3-5×10^4 cells/ml, and 100 μL per well was added to a 96-well culture plate. The cells were cultured for 6 days under normal conditions, and then, 20 μL of 1% MTT was added to each well for 5 h every 24 h for 6 successive days. To stop the culture, 150 μL of dimethyl sulfoxide (DMSO) was added to each well, and the absorbance of each well was detected with a microplate reader at a wavelength of 570 nm. Six samples from each group were measured, and a cell growth curve was generated with the average of the daily absorbence values.

2.3 Flow cytometric analysis of the cell cycle

Cells that had been passaged three times were trypsinized with 0.25% trypsin-EDTA and pelleted by centrifugation at 1000 rpm for 5 min The pellets were washed twice with cold PBS and were fixed in 70% cold ethanol at 4°C overnight. After fixation, the cells were washed with cold PBS and incubated in a solution of 20 μg/ml RNase

A and 50 μg/ml propidium iodide (PI) for 30 min at room temperature. Stained cells were analysed by fluorescence activated cell sorting (FACS) with a flow cytometer. The proportion of cells in G_0/G_1, S, and G_2/M phases was calculated with the CellQuest software (Becton-Dickinson). The proliferation index was calculated according to the following formula: PI (%) = $([S+G_2/M]/[G_0/G_1+S+G_2/M])\times100\%$.

2.4 Image cytometric DNA ploidy analysis

Automated quantitative DNA image cytometry (DNA-ICM) was used for DNA ploidy analysis. Approximately 5-10 × 10^3 cells were trypsinized with 0.25% trypsin-EDTA and pelleted by centrifugation at 1000 rpm for 5 min. The pellets were collected and fixed in PreservCyt (Heer, Wuhan, China) and were then used to generate slides using the Feulgen method under temperature-controlled conditions; these conditions were similar to those that have been reported in previous studies [18]. The SPICM-DNA system was used for the automatic classification of cells, measurement of DNA ploidy and analysis of aneuploidy, which was purchased from the Heer Company (Wuhan, China). For each slide, all of the cell nuclei on the slide were scanned, and nuclear images were collected to generate a nuclear DNA image gallery. The DNA content of each cell nucleus was measured. A DNA index (DI) of 1 corresponds to 7 pg of diploid DNA, and a DI of 2 corresponds to 14 pg of tetraploid DNA. When the DNA indices range from 0.9-1.1, the sample is considered to be diploid, and when the DNA indices range from 1.9-2.1, the sample is considered to be tetraploid. When the DI falls outside of this range, the cell is considered to be aneuploid.

The procedures described above were performed with three biological replicates.

2.5 Statistical analysis

The SPSS (Statistical Package for the Social Sciences) for Windows version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Quantitative data are expressed as the mean ± standard deviation (SD), and the significance of the differences between groups was evaluated with one-way analysis of variance (ANOVA). Cell growth curves were analysed with GraphPad Prism software. Significance was assumed if P was <0.05.

Ethical approval: The research related to human use has been complied with all the relevant national regulations and institutional policies, and has been approved by the authors' institutional review board committee

3 Results

3.1 Growth features of human cervical precancerous cells

The growth curves are shown in figure 1. The absorbance values are not shown. As expected, statistically significant differences were observed among the cervical precancerous, NUC and Caski cells (P<0.01). The curve of the cervical precancerous cells did not have the typical 'S' type and resembled that of the Caski cells. After re-seeding, the cervical precancerous cells were in an adaptive phase for 2 days. Then, the cells began to expand rapidly as they progressed into a logarithmic phase of growth. Five days later, the cell counts reached their highest level, which was followed by a plateau phase. According to the growth curve, the population doubling time of the precancerous cells was approximately 48-50 h, which was much faster than that of the NUC keratinocytes (P<0.01). This result showed that the proliferative capacity of the cervical precancerous cells was close to that of the cancerous cells at the cellular level.

3.2 Flow cytometric analysis of the cell cycle

The FACS profiles of the NUC, cervical precancerous and Caski cells are shown in figure 2. The distribution of the cells in each phase of the cell cycle is shown in table 1. Each value is presented as the mean ± SD. The data are representative of at least three independent experi-

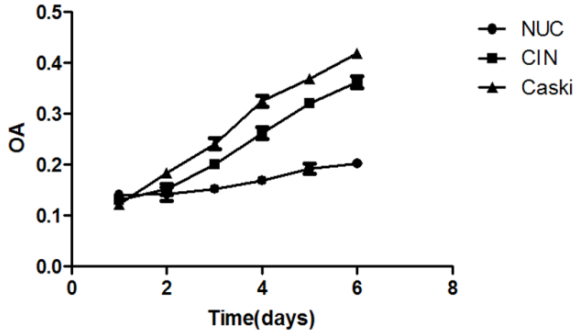

Figure 1: Measurement of cell growth curves with MTT.

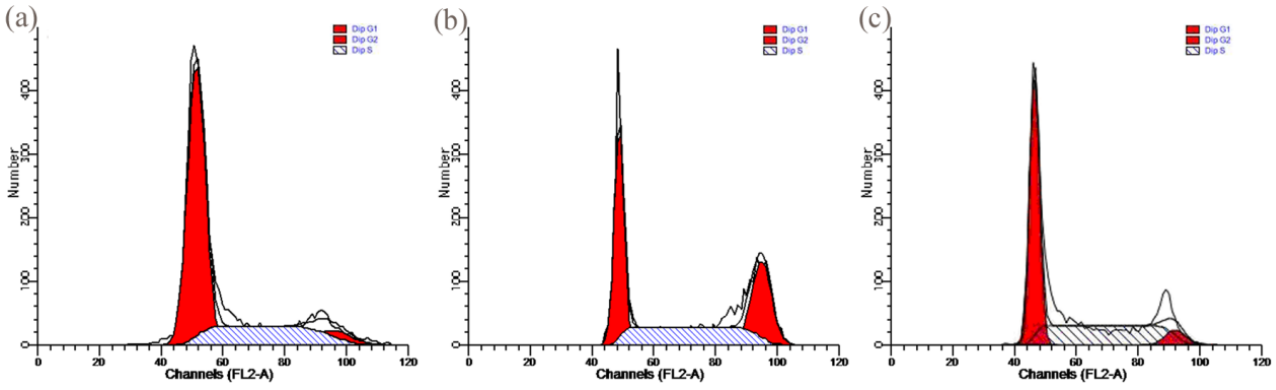

Figure 2: Cell cycles analyzed by flow cytometry. (a) DNA histogram from NUC cell group showed: G0/G1 = 67.07%, S = 25.92%, G2/M = 7.02%. (b) DNA histogram from precancerous cell group showed: G_0/G_1 = 38.03%, S =32.74 %, G_2/M = 29.23%. (c) DNA histogram from Caski cell group showed G_0/G_1 = 52.50%, S = 41.75%, G_2/M =5.75 %.

Table 1: The percentage of cells in G_0/G_1, S and G_2/M phases among NUC, precancerous and Caski cell groups ($\chi \pm S$).

Parameters	NUC (%)	Precancerous (%)	Caski (%)	P value
G_0/G_1	60.18±1.07	39.16±1.03	53.00±0.44	<0.01
S	28.50±0.14	32.74±1.18	39.10±3.24	<0.01
G_2/M	11.33±1.20	28.07±1.30	7.91±2.87	<0.01 *
PI	39.82±1.07	60.84±1.03	47.01±0.44	<0.01

* No significant difference was observed between NUC and Caski cell groups according to multiple comparisons (P=0.75).

ments. The percentages of cells in each of the cell cycle stages were comparable among the groups. These results showed that almost all of the cells in the 3 groups demonstrated the ability to proliferate. However, an analysis of the cell cycles indicated that there were significant differences among the NUC, cervical precancerous and Caski cell groups (P<0.01). In contrast to the NUC cells, greater numbers of the cervical precancerous cells were in S phase, whereas more of the Caski cells were in S phase than that in any other cell type (P<0.01). Interestingly, the proliferation index of the cervical precancerous cell group was significantly higher than that of the Caski cell group due to the significantly higher proportion of cervical precancerous cells in G_2/M phases compared with that of the Caski cells (P<0.01).

3.3 Image cytometry DNA ploidy

The DNA image cytometry profiles of the NUC, cervical precancerous and Caski cells are shown in figure 3. The

Table 2: Comparison of DNA ploidy among the NUC, precancerous and Caski cell groups ($\chi \pm S$).

DNA ploidy	NUC (%)	Precancerous (%)	Caski (%)	P value
Diploid	75.17±0.88	65.52±1.56	62.73±1.28	<0.01
Aneuploid	9.96±0.09	23.06±0.04	23.43±0.85	<0.01*
Quadraploid	15.04±0.87	12.01±0.75	13.85±0.43	<0.01†

* No significant difference was observed between precancerous and Caski cell groups according to multiple comparisons (P=0.392).
† No significant difference was observed between NUC and Caski cell groups according to multiple comparisons (P=0.084).

association between the cell type and DNA ploidy is shown in table 2. These results showed that aneuploid cells were detected in each group. The proportion of cells with DNA aneuploidy in the cervical precancerous and Caski cell groups was significantly higher than that in the NUC cell group (P<0.05). However, the difference in the proportion of cells with DNA aneuploidy between the Caski cell group and the cervical precancerous cell group was not statistically significant (P=0.392).

4 Discussion

In this study, we found that precancerous and cancerous cells of the cervix demonstrate a similar proliferative capacity at the molecular level. It is widely assumed that, in theory, cancerous cells should have a significantly greater ability to proliferate than that of precancerous cells; unfortunately, almost no research has been conducted on the proliferation capacity of precancerous cells in vitro. To the best of our knowledge, studies on cervical precancerous lesions have been confined to clinical

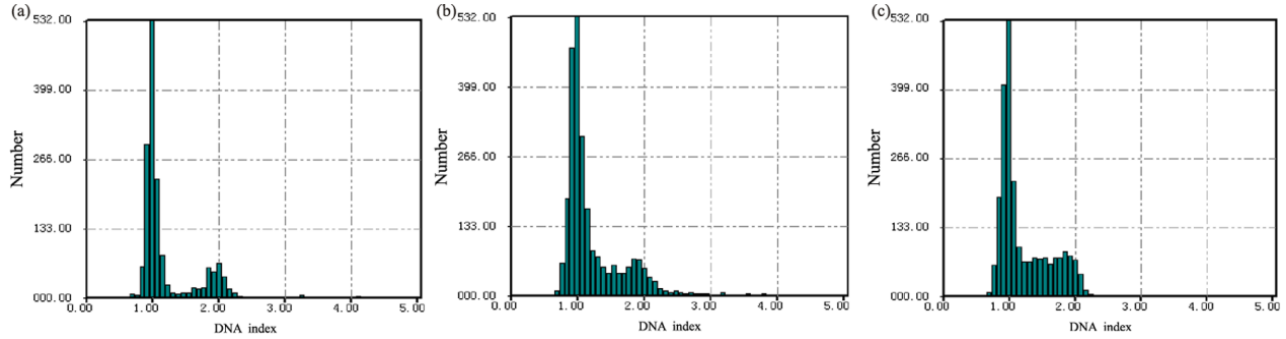

Figure 3: Cell cycles analyzed by DNA image cytometry. (a) DNA ploidy from NUC keratinocyte group showed: Diploid= 75.12%, Aneuploid= 9.47%, Quadploid=15.41%. (b) DNA ploidy from precancerous cell group showed: Diploid= 67.24%, Aneuploid= 21.49%, Quadploid= 11.27%. (c) DNA ploidy from Caski cell group showed: Diploid= 62.73%, Aneuploid= 23.42%, Quadploid= 13.85%.

trials [4, 5, 7]. Our study is the first to explore the in vitro cell proliferation capacity of precancerous lesions of the cervix at the cellular and molecular levels.

An MTT assay is a common method that is used to evaluate the cell proliferation rate. From the growth curve presented in our study, we observed that the cervical precancerous cells exhibited higher rates of proliferation compared with those of the NUC cells and slower rates compared with those of the Caski cells, which only exhibited a transient adaptive phase, similar to other cancerous cell lines that are described in the literature [19, 20]. Unexpectedly, the growth curve of the cervical precancerous cells in the present study was similar to that of the primary cultured cervical carcinoma cells according to a preliminary experiment (data not shown) and as described in previous studies [9, 11]. This is consistent with the observation that cancerous cervical lesions are often surrounded by precancerous lesions that have an intact basement membrane and that cultured primary purified cervical cancerous cells from cervical carcinoma tissues are impractical and very difficult to culture. Additionally, the biological characteristics of the cultured primary cells are slightly different from those of cell lines, which may be related to the culture conditions, but cultured primary cells are more representative of primary human tumours than the cell lines. Therefore, the growth curve presented in our study may represent the true proliferation capacity of uterine cervical precancerous cells.

The cell cycle comprises interphase, which consists of the G1, S, and G2 phases, and the mitotic (M) phase [21], and the cell cycle can be analysed by different methods, including flow cytometry (FCM) and image analysis, according to cellular DNA content. In the past several years, FCM has been regarded as a powerful tool that is well suited for the rapid analysis of cellular DNA content and proliferative activity. Therefore, we first analysed the SPF using FCM in this study. For the first time, we confirmed that the SPF of the cervical precancerous cells was significantly higher than that of the NUC cells, but it was still lower than that of the Caski cells at the molecular level. Interestingly, the proliferation index, which also indicates the cell proliferation activity, was higher in the cervical precancerous cells than in the Caski cells due to the significantly higher proportion of cervical precancerous cells in the G2/M phases compared with those of the Caski cells. This phenomenon was consistent with the hypothesis that oncogenic HPV DNA was integrated into the human genome and deregulated the transcription of the viral oncogenes E6 and E7 and caused chromosomal instability [22]. DNA damage could induce S and G2/M cell cycle arrest [23].

Chromosomal instability and aneuploidy are similar but are not synonymous; chromosomal instability is the process that leads to chromosome copy number alterations, and aneuploidy is the result of that process [24]. It was reported that image cytometry has been shown to be superior to FCM in the identification of aneuploid cases due to technical and methodological problems [11, 25]. Moreover, the flow cytometric S-phase fraction (SPF) has not been generally used in standard practice in recent years. Meanwhile, the availability of image-processing instruments gives the pathologist a direct visualization of the cell population being studied so that DNA measurement may be performed; this provides the ability to simultaneously examine the amount of DNA and morphologic features and to identify any correlations [26]. Therefore, the automated quantitative image analysis of DNA aneuploidy was also performed in this study, and we observed that the proportion of DNA aneuploidy in the cervical precancerous cells was much higher than that in the NUC cells but was very similar to that in the Caski cells, which was we had not expected. Some studies have shown that automated DNA ICM technology might increase the sensitivity of the cytologic detection of dysplastic and can-

cerous cells in several types of cancers; these cells in turn could be used as a marker of progression [18, 27]. However, the significance of DNA aneuploidy has been confined only in clinical studies in the literature and is not yet fully understood at the molecular level. Generally, aneuploidy is a hallmark of cancer cells, and detecting aneuploid cells is necessary to detect cancer cells [27, 28]. However, data from our study indicated that DNA aneuploidy not only exists in cancerous cells but is also a feature of precancerous and normal cells; therefore, we deduced that the occurrence of aneuploid cells is only a sign of a proliferative state and cannot be used as a marker to distinguish precancerous cells from cancerous cells, but aneuploidy could be used to differentiate precancerous and cancerous cells from normal cells. Meanwhile, the predictive significance of aneuploidy for pre-cancer and cancer outcomes has also been questioned in the present study.

This study has several limitations that need to be considered during the interpretation of our findings. First and foremost, Caski cells, but not primary cervical carcinoma cells, were designated as the positive control group. To some extent, slight differences in biological characteristics may be present between cell lines and primary cells; moreover, it is generally accepted that primary cells, rather than cell lines, may be more reflective of the biological characteristics of cells in vivo. However, precancerous and cancerous lesions often coexist on the cervix; therefore, it was very difficult and nearly impossible to cultivate highly purified primary cervical cancerous cells. Additionally, BrdU and EdU cell proliferation assays, which have been regarded as the most accurate and traditional methods for the direct measurement of DNA synthesis, were not adopted in this study to measure cell proliferation. This is because the use of radioisotopes in these assays is harmful to experimenters, and these methods are not widely used in clinical practice.

In conclusion, we demonstrated that the proliferative activity of the cells in human precancerous lesions of the cervix, as evaluated by cell growth curves, flow cytometry cell cycle analysis and DNA aneuploidy, was much higher than that of NUC cells. However, contrary to our expectations, we showed for the first time that precancerous and cancerous cells of the cervix demonstrate a similar proliferative capacity at the molecular level. Therefore, we conclude that the surrounding environment of the cells may play an important role in the development of cervical cancer. The results of this study might provide an important basis for future studies of precancerous and cancerous lesions of the cervix.

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Data Availability: The data used to support the findings of this study are mainly included within the article, and the underlying data are available from the corresponding author upon request.

Conflict of interest: The authors have no conflicts of interest to declare.

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