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ORIGINAL ARTICLE

Effects of Nordy on the proliferation, differentiation, and apoptosis of HPV16 subgene-immortalized human endocervical cells*

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Abstract

Objective The aim of this study was to investigate the effects of Nordy on the proliferation, differentiation, and apoptosis of HPV16 subgene-immortalized human endocervical cells (H8 cells).

Methods After treatment with Nordy, H8 cell proliferation was evaluated using the MTT assay. The effects of Nordy on the cell cycle and apoptosis of H8 cells were analyzed by flow cytometry (FCM) and the Annexin V-FITC method. H8 cell MCM5 expression was detected by immunocytochemistry. Morphological changes were observed by light and electron microscopy. Telomerase activity was evaluated by TRAP-ELISA.

Results We found that 10 μmol/L–100 μmol/L Nordy significantly inhibited H8 cell proliferation. After treatment with Nordy, H8 cells were blocked in the G_0/G_1 phase, and the rate of cell apoptosis increased significantly. Cells differentiated toward innocuousness, and MCM5 expression and telomerase activity notably decreased.

Conclusion Nordy was observed to inhibit proliferation and promote apoptosis in H8 cells. Nordy also induced H8 cell differentiation; this effect may have been achieved by blocking the cell cycle and decreasing telomerase activity.

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Key word: induced differentiation; cervical cancer; immortalization; HPV

Nordy is a new artificial compound synthesized based on the structure of nordihydroguaiaretic acid (NDGA). NDGA is a natural product purified from *Larrea divaricata* and *Guaiacum officinale* plants, and is used as a natural lipoxygenase inhibitor. Recent research has revealed that NDGA has tumor-inhibiting properties [1-2]. However, it is difficult to extract NDGA from plants, and the purity of such extractions is very low. Based on the structure of NDGA, we improved and synthesized a new compound-Nordy. This compound has more effective anti-tumor properties and lower drug toxicity. It is easy to synthesize in laboratory conditions, with very high purity of up to > 99%. The effects of Nordy on immortalized cervical cells have not previously been reported. In this study, we investigated the effects of Nordy on the proliferation, differentiation, and apoptosis of human papilloma virus (HPV) 16 subgene-immortalized human endocervical cells (H8 cells-precancerous state cells) *in vitro*, along with its possible mechanisms.

Materials and methods

Materials and reagents

The H8 cell line was provided by the Chinese Academy of Medical Sciences. Nordy was synthesized by the Institute of Pathology of the Third Military Medical University, and 3-(4, 5-demethy-2thiazolyl)-2, 5-dephenyl-2h-tetrazolium-bromid (MTT) and DMSO were obtained from Sigma (USA). RPMI-1640 was purchased from Gibco (USA), calf serum (CS) was from Shijiqing Corp (Hangzhou, China), and the PCR-ELISA kit was purchased from Huamei Corp (Shanghai, China).

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The mouse monoclonal MCM5 was from Lab Vision (USA). The immunohistochemistry SP kit and DAB were obtained from Zhongshan Corp (Beijing, China). At 80% confluence, the cells were washed once with PBS and incubated for 24 h in 0.05% calf serum-RPMI, followed by treatment with Nordy.

Cell culture

H8 cells were maintained in RPMI-1640 containing 10% FBS and an antibiotic-antimycotic. The cells were cultured in a humidified incubator containing 5% CO_2 at 37°C.

H8 cell proliferation (MTT assay)

Cell growth was measured using a modified MTT assay. Approximately 5×10^4 cells (in 200 μL) were cultured in 96-well plates and incubated overnight. Cells were then treated with 10 μmol/L, 50 μmol/L, 75 μmol/L, or 100 μmol/L Nordy for 24 h, 48 h, 72 h, or 96 h. Twenty microliters of MTT were added to each well and the cells were further incubated at 37°C for 4 h. Cell supernatant was removed, and 200 μL DMSO in isopropanol was added to each well. The spectrometric absorbance at 570 nm (A_{570}) was measured on a microplate reader (Sigma, USA). The negative control well contained medium only. The ratios of the absorbance of treated groups relative to those of the control group were calculated and expressed as percentage of growth inhibition.

Immunocytochemistry

H8 cells that had been treated with 100 μmol/L Nordy for 72 h were fixed with stock acetone. Detection was carried out as described in the kit protocol. MCM5-positive cells were defined as those with an aggregation of brown particles in the cell nucleus. The rate of positive expression = (positive cells / 500 cells) × 100%.

Flow cytometry

Cells were seeded into 50 mL plastic culture flasks at a density of 5×10^4 cells/mL, in the absence or presence of 10 μmol/L, 50 μmol/L, 75 μmol/L, or 100 μmol/L Nordy. After treatment for 72 h, 1×10^6 cells were harvested, pelleted, and washed in phosphate-buffed saline (PBS), fixed with 70% cold alcohol at 4°C and stained with propidium iodide (ClonTECH, Japan). Specimens were analyzed by FASCan (USA) to estimate the percentage of cells in each phase of the cell cycle.

Annexin V-FITC

Cells were seeded into 50-mL plastic culture flasks at a density of 5×10^4 cells/mL, in the absence or presence of 100 μmol/L or 200 μmol/L Nordy. After treatment for 48 h, $1–5 \times 10^5$ cells were harvested. Two hundred and fifty microliters of 2 × binding buffer were mixed with 250 μL sterile deionized water, 500 μL of cells resuspended in 1× binding buffer, and 1 μL Annexin V-FITC, and the reaction was allowed to progress for 5 min, FCW (Ex=488 nm; Em = 530 nm).

Morphological changes

Cells were seeded in 50-mL plastic culture flasks, in the absence or presence of 100 μmol/L Nordy. Cells were incubated at 37°C, in 5% CO_2 for 72 h. Routine HE staining was performed and observed under a light microscope. Cells were collected into 1.5-mL EP tubes and fixed using 1.5 mL of 2.5% paraformaldehyde for 5 min. Sections were prepared and photographed using a Hitachi 600 Electron Microscope (Japan).

TRAP-ELISA assay

Cells were seeded into 50-mL plastic culture flasks at a density of 5×10^4 cells/mL, in the absence or presence of 16 μmol/L Nordy. After treatment for 48 h, 72 h, or 96 h, 1×10^6 cells were harvested. Telomerase was extracted and its activity evaluated using a TRAP-ELISA assay, according to the instructions in the kit.

Statistical analysis

Unless specified, all experiments were performed at least three times and representative results were presented. Data were analyzed using SPSS12.0 statistical software. An unpaired dependent *t*-test was used to determine the significance of the difference between the two groups. ANOVA was used to determine the statistical significance of the differences among multiple groups; the interclass ambi-comparison was the Bonferroni method; and immunocytochemistry was analyzed with a rank-sum test. For comparison of protein expression levels, an independent *t*-test was used. Statistical significance was set at $P < 0.05$.

Results

Effects of Nordy on H8 cell proliferation

The growth of H8 cells in the treated group was significantly inhibited in a dose- and time-dependent manner compared to that of the control group. After treatment for 72 and 96 h, the inhibition rate was in the range of 11.3%–45.1% and 15.2%–48.6%, respectively. Statistical analysis indicated that the differences between treatment times and doses were significant ($P < 0.05$, Fig. 1)

Effects of Nordy on MCM5 expression

After treatment with 100 μmol/L Nordy for 72 h, the MCM5 expression rate decreased from 70.7 ± 0.45% to

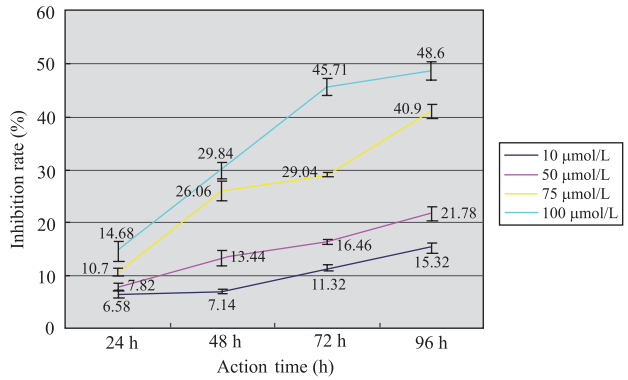
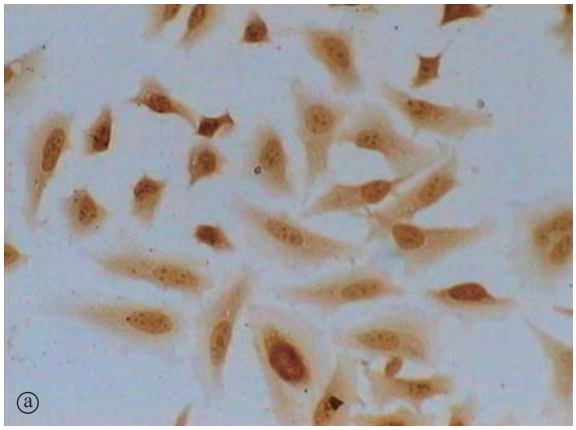


Fig. 1 The effect on H8 cells proliferation by varied concentration Nordy



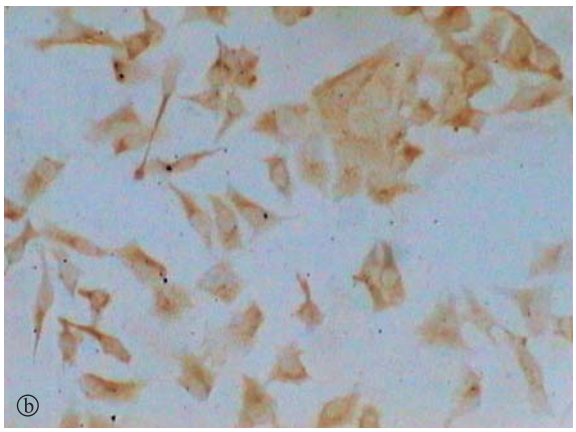
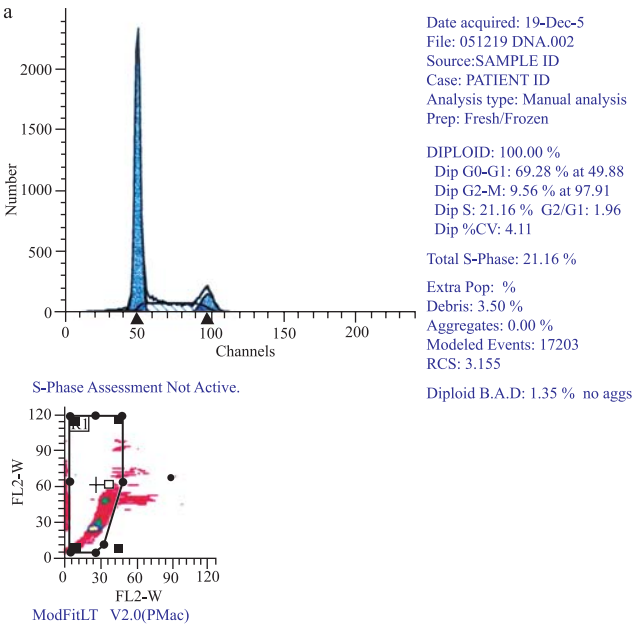


Fig. 2 The expression of mcm5 in control group and treated group. (a) Positive expression of mcm5 of control group with (SP×200). (b) Weaken expression of mcm5 after treated Nordy for 72 h (SP × 200)

12.69 ± 1.71%. The difference in expression rate between the control group and the treated group was statistically significant ($P < 0.05$; Fig 2) .

Effects of Nordy on the cell cycle

The effects of Nordy on the cell cycle were shown in Table 1. After treatment with 10 μmol/L, 50 μmol/L, 75 μmol/L, or 100 μmol/L Nordy for 72 h, the percentage of cells in the G_0/G_1 phase significantly increased compared to that of the control group; however, the percentage of cells in the S and G_2/M phases significantly decreased. This suggested that H8 cell growth was arrested in the G_0/G_1 phase (Table 1, Fig 3).



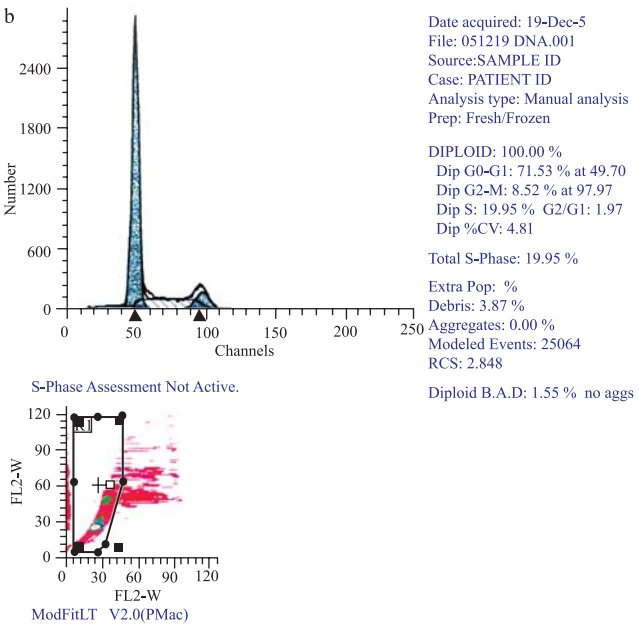


Fig. 3 The effects of different concentration of Nordy on the cell cycle. (a) 50 μmol/L Nordy for 72 h. (b) 100 μmol/L Nordy for 72 h

Table 1 The effects of Nordy on cell cycle of H8 cells (%, mean ± *s*)

Cell cycle	Control	10 μmol/L	50 μmol/L	75 μmol/L	100 μmol/L
G_0/G_1	57.05 ± 0.175	58.93 ± 1.982	68.46 ± 0.683	70.17 ± 0.811	72.6 ± 3.094
S	27.55 ± 0.447	26.18 ± 1.442	21.27 ± 0.658	20.42 ± 0.891	18.9 ± 1.956
G_2/M	15.39 ± 0.277	14.85 ± 0.532	10.26 ± 0.674	9.07 ± 0.436	8.48 ± 1.215

Table 2 The effects of Nordy on cell apoptosis of H8 cells (mean ± s)

	Control	100 μmol/L	200 μmol/L
mean ± s	3.478 ± 1.519	12.322 ± 0.786	24.330 ± 3.228
P	0.8214	< 0.05[a]	< 0.01[b]

[a] $P < 0.05$, vs100 μmol/L Nordy group; [b] $P < 0.01$, vs 200 μmol/L Nordy group

Table 3 Effects of Nordy on the activity of telomeras (mean ± s)

Activity of telomerase (OD value)	48 h	72 h	96 h
Control group	0.617 ± 0.009[c]	0.607 ± 0.040[c]	0.616 ± 0.025[c]
16 μmmol/L Nordy	0.424 ± 0.007[ab]	0.315 ± 0.009[ab]	0.138 ± 0.007[ab]

[a] $P > 0.05$, vs control; [b] $P < 0.05$, treatment of different time; [c] $P < 0.05$, control groups of different time

Effects of Nordy on cell apoptosis

By using the Annexin V-FITC method, the apoptosis rates of cells treated with 100 μmol/L and 200 μmol/L Nordy for 48 h were found to be 12.322 ± 0.786% and 24.330 ± 3.228%, respectively, and the apoptosis rate of the control cells was 3.478 ± 1.519%. After treatment with Nordy, the apoptosis rate of H8 cells increased significantly ($P < 0.05$, $P < 0.01$; Table 2, Fig 4).

Morphological changes

Using light microscopy, we observed that control H8 cells were large and polygonal with dark nuclei. Many nuclear mitoses were observed (Fig. 5a). After treatment with Nordy for 72 h, the cells became smaller and more regular in shape, and the number of nuclear mitoses decreased (Fig. 5b). The cell nuclei became smaller and more regular in shape, as observed by electron microscopy. The quantity of heterochromatin increased, and some mature organelles such as mitochondria and endoplasmic reticulum were clearly visible. Cell apoptosis was frequent, and there was a notable increase in apoptotic bodies (Fig. 6).

Effects of Nordy on telomerase activity

Telomerase activity was remarkably high in the control cells. After treatment with 16 μmol/L Nordy for 48 h, 72 h, and 96 h, telomerase activity in the treated cells decreased in a time-dependent manner. The differences between the control group and the treated groups with different treatment times were statistically significant ($P < 0.05$; Table 3).

In our experiment, we first determined that Nordy inhibited proliferation and promoted apoptosis in H8 cells. Nordy was also observed to induce H8 cell differentiation. This effect may have been achieved by blocking the cell cycle and decreasing telomerase activity.

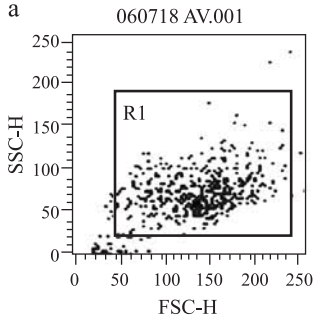
Discussion

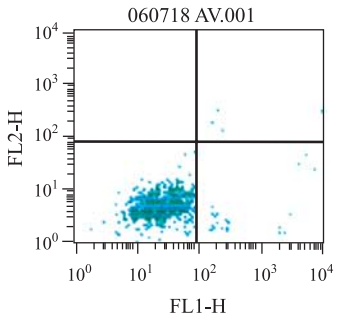
OCancer of the cervix is a gynecology-related tumor. The incidence of this disease is ranked the second highest among malignant tumors in females. HPV is a major cause of cervical cancer. Eighteen high-risk subtypes, especially HPV16, are regarded as major causes of cervical cancer by the international medical science community [3]. HPV genes can integrate into the chromosomes of host cells, activating the relevant oncogenes and virus-transforming proteins. Meanwhile, P53 or Rb protein in host cells are also inactivated, leading to uncontrollable growth of host cells [4]. HPV genes E6 and E7 can change the growth mode of host cells, causing atypical hyperplasia of host cells and cervical intra-epithelial neoplasia, and even cancer of the cervix [5–7]. In this study, we transfected HPV16 E6/E7 subgenes into an H8 cell line *in vitro* and created immortalized cells. These subgene-immortalized human endocervical cells were therefore regarded as precancerous-state cells.

The MTT assay results indicated that Nordy significantly inhibited H8 cell proliferation, in a time- and dose-dependent manner. Meanwhile, we found that Nordy disturbed the H8 cell cycle, increasing the percentage of cells in G_0/G_1 phase and decreasing the percentage of cells in S phase, as observed by FCM. This effect suggests that Nordy arrested H8 cells in G_0/G_1 phase, inhibited DNA synthesis, and slowed down the velocity of cell division. MCM5, a nuclear protein expressed in the proliferation phase, plays a vital role in cell DNA reproduction, and is therefore regarded to be a good biological maker of cell proliferation [8-10]. We found that Nordy treatment notably reduced MCM5 expression of MCM5, indicating a corresponding decrease in cell proliferation activity. This was in accordance with FCM results.

Cell apoptosis plays an important role in tumor

a





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Quad	% Gated
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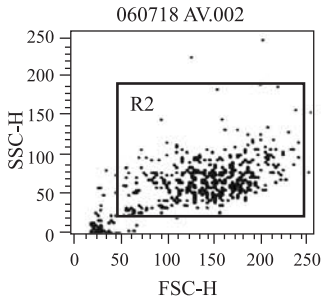
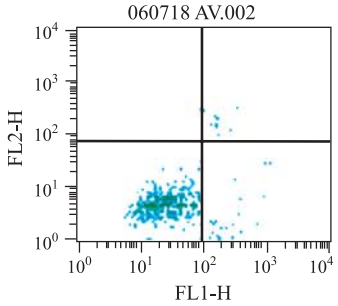
UR	0.20
LL	96.90
LR	2.82





File: 060718 AV.002

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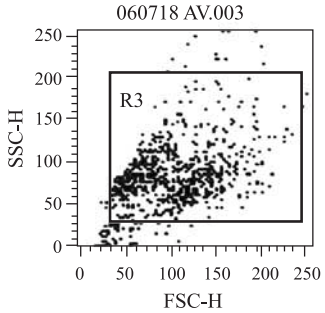
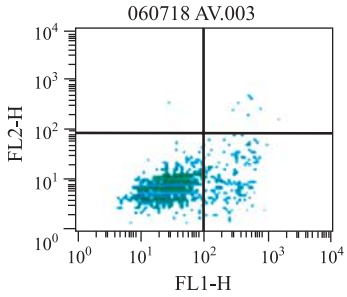
UR	0.83
LL	94.86
LR	4.18





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Quad	% Gated
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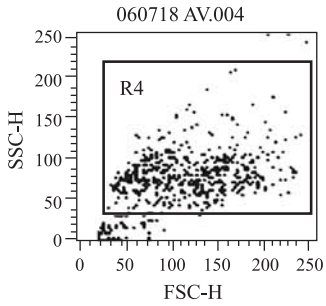
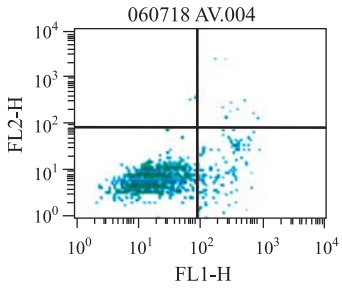
LL	87.75
LR	11.30





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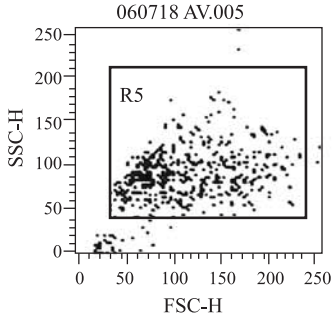
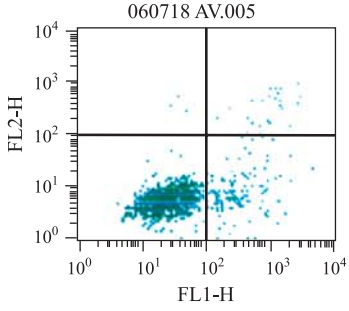
UR	3.73
LL	83.98
LR	11.98





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LR	12.37

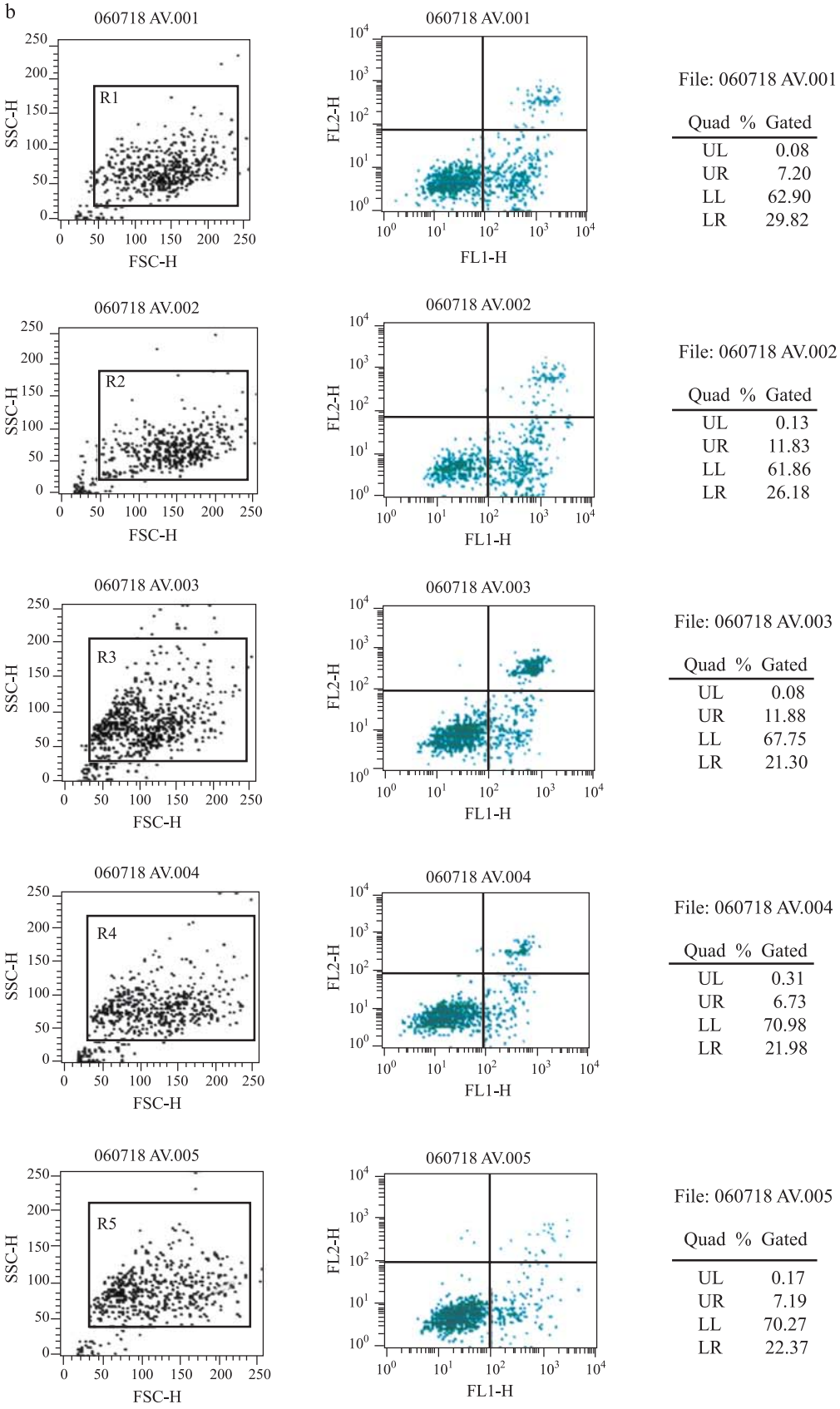

Fig. 4 The effects of different concentration of Nordy on cell apoptosis. (a) 100 μmol/L Nordy for 48 h. (b) 200 μmol/L Nordy for 48 h.

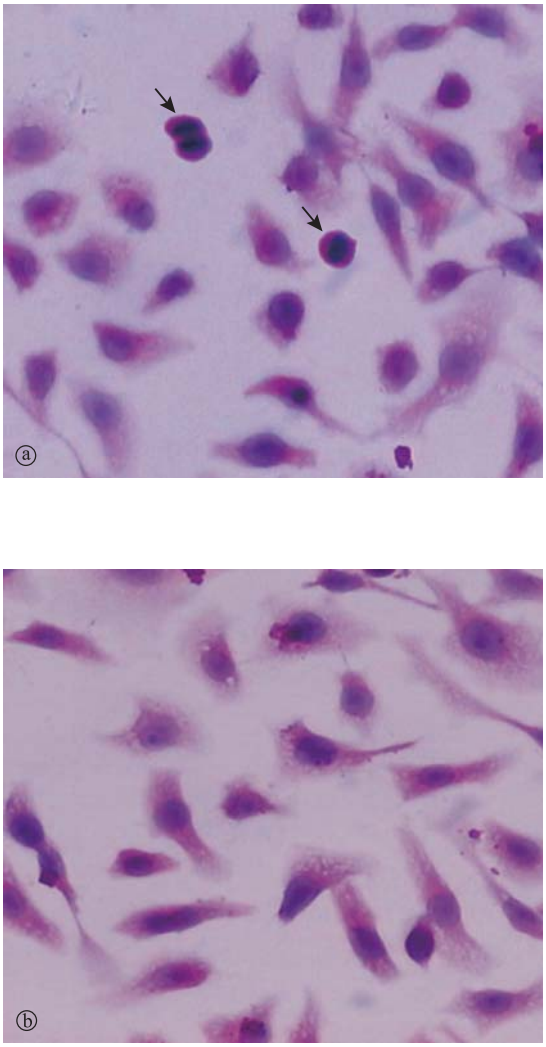

Fig. 5 The microstructural changes of cell were observed by optical microscope. (a) Control group cell, Karyomegaly and anachromasis (HE × 400); (b) H8 cell after treated with Nordy for 72 h (HE × 400)

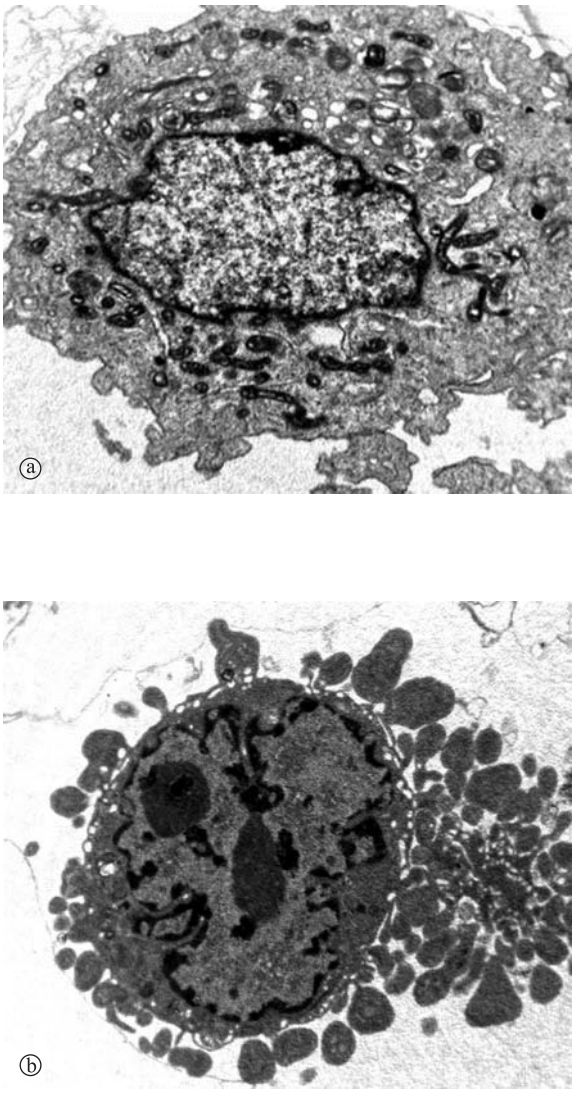

Fig. 6 The Ultramicrostructural changes of Cell were observed by electron microscope. (a) H8 cell after treated with Nordy for 72 h, mature, nuclear, chondrosome, endoplasmic reticulum (TEM × 10000) (b) H8 cell after treated with Nordy for 72 h, apoptoticbodys (TEM × 7000)

pathogenesis. Ways to induce tumor cell apoptosis therefore became a smart strategy and direction for tumor treatment [11].In our study, through the Annexin V-FITC method, we observed that the apoptosis rate of H8 cells increased significantly after treatment with Nordy, and that apoptotic bodies notably increased, as observed by electron microscopy. These factors indicate that Nordy promoted H8 cell apoptosis.

Cytodifferentiation is demonstrated by changes in morphology and function. Using light microscopy and electron microscopy, we observed that H8 cells became more morphologically mature after treatment with Nordy. Cell size and nuclear size decreased, as did the ratio of nucleus to cytoplasm and number of nucleoli. Heterochromatin increased, and some mature organelles, such as mitochondria and endoplasmic reticulum, were visible.

Several recent reports have described the presence of telomerase activity not only in cervical tumor lesions but also in premalignant lesions [12]. Wang [13] reported that telomerase activity in cells from patients with CIN was higher than in cells from patients with cervicitis. Moreover, there was a significant correlation between the severity of cervical lesions and the signal intensity of telomerase activity. Telomerase activity might be considered to be a marker of early diagnosis and progression of cervical neoplasia [14–15]. In this study, after treatment with Nordy for 48–96 h, telomerase activity decreased in a time-dependent manner. This result shows

that Nordy downregulated telomerase activity in H8 cells.

Our experiments showed that Nordy inhibited the proliferation of HPV16 subgene-immortalized human endocervical cells, induced cell differentiation, and promoted apoptosis. This may be via a mechanism whereby Nordy inhibited DNA reproduction in the S phase, and downregulated H8 cell telomerase activity. These results suggest that Nordy may be a promising new therapeutic for tumors or precancerous lesions.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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