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Effects of deuterium-depleted water on proliferation and differentiation of human lung cancer A549 cells and its mechanism

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Abstract: Objective To study the effect of deuterium-depleted water (DDW) on the proliferation and differentiation of human lung cancer A549 cells and to explore its mechanism. Methods

A549 cells were divided into an observation group and a control group. The cells were cultured in a medium prepared with DDW containing 50 ppm deuterium and a medium prepared with normal water for 30 days, respectively. The cell morphology of the two groups was observed under an inverted microscope, and the cell growth was determined by CCK-8 method within 48 hours. The cells in the observation group and the control group were first cultured in serum-free DDW medium and normal water medium for 48 h to synchronize the cell growth, and then added with DDW medium containing 10% serum and normal water medium for further culture.

The proportions of cells in the G1, S and G2 /M phases of the cell cycle at 36 and 48 h in the two groups were determined by propidium iodide staining and flow cytometry. The cell lysates of the two groups were taken to extract total cell protein. The expressions of mitogen-activated extracellular signal-regulated kinase (MEK1) and epidermal growth factor receptor (EGFR), the phosphorylation levels of serines 217 and 298 of MEK1, and the phosphorylation levels of tyrosines 1016 and 1110 of EGFR were detected by Western blot. The specific protein markers of alveolar type ÿ epithelial cells AQP5 and T1a and the specific protein markers of alveolar type ÿ epithelial cells SPB, SPC1, SPC2, Expression of CFTÿ. Results The cells in the control group were cubic, with close contact and accumulation between cells, while the cells in the observation group were spindle-shaped, elongated, and with loose contact between cells. The OD values of the observation group at 36 and 48 h were lower than those of the control group (all $P < 0.05$). After serum starvation and then stimulation for 36 h, the proportion of cells in the G1 phase in the observation group was 71.75%, which was higher than 57.01% in the control group; the proportion of cells in the S phase in the observation group was 22.43%, which was lower than 35.88% in the control group (all $P < 0.01$); there was no statistically significant difference in the cell cycle ratio between the two groups at 48 h.

There was no significant difference in the expression of MEK1 and EGFÿ and the levels of tyrosine phosphorylation at positions 1016 and 1110 of EGFÿ between the two groups. The levels of serine phosphorylation at positions 217 and 298 of MEK1 were decreased in the observation group ($P < 0.01$ for both groups). The level of SPC2 in the observation group was higher than that in the control group ($P < 0.01$). Conclusion Long-term culture of A549 cells with DDW can significantly inhibit their cell proliferation and differentiation. The mechanism may be related to reducing the phosphorylation level of MEK1, blocking the G1 /S phase transition of the cell cycle, inducing the transformation of A549 cells from type II alveolar epithelial cells to type I alveolar epithelial cells, and improving the degree of cell differentiation.

Keywords: lung cancer; deuterium-depleted water; cell cycle; mitogen-activated extracellular signal-regulated kinase; cell differentiation doi: 10.3969/j.issn.1002-266X. 2017ÿ 06ÿ 001 Chinese

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Effects of deuterium-depleted water on proliferation and differentiation of lung cancer A549 cells

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Abstract: Objective To study the effects of deuterium-depleted water (DDW) on human lung cancer cell line A549 and to explore the underlying molecular mechanisms. Methods A549 cells were randomly divided into two groups: the observation group and the control group, which were seperately cultured in the medium prepared with 50 ppm DDW and in the medium prepared with normal water for 30 d. The morphology of cells was observed under an inverted microscope. Cell growth was measured using the colorimetric cell counting kit-8 (CCK-8) reagent to compare the growth rate of cells be-tween the two groups. The cells in the two groups were synchronized by serum starvation and then were separately stimula-ted with medium containing 10% serum of DDW and medium containing normal water. At 36 and 48 h after the stimula-tion, cells from each group were trypsinized, stained with propidium iodide (PI) and then were analyzed by fluorescence-activated cell sorting (FACS) to determine the percentages of cells distributed in G1, S and G2 /M phases during cell cy-cle. Total proteins were extracted from cells cultured in normal medium or DDW medium. Western blotting was conducted to detect the expression levels of MEK1 and EGFR, phosphorylation of serine 217 and serine 298 on MEK1, and phospho-rylation of tyrosine 1016 and tyrosine 1110.

The expression levels of several alveolus-specific protein markers (type Ⅰ alveolus markers: AQP5 and T1a, type Ⅱ alveolus markers SPB, SPC1, SPC2 and CFTR) in cells of the two groups were also compared. Results In the control group, the cells were cuboidal and in close contact with accumulation of cells. In the observation group, the cells were spindle, and intercellular contact was loose with cell elongation. The OD of the obser-vation group was lower than that of the control group (all $P < 0.05$) . After the serum starvation, cells were stimulated for 36 h, the percentage of cells in G1 phase of the observation group was 71. 75% , which was higher than that of the control group (57. 01%) , the percentage of cells in S phase was 22. 43% , which was lower than that of the control group (35. 88%) , (all $P < 0.05$) . There was no significant difference in cell cycle ratio between the two groups at 48 h. No sig-nificant difference was found in the expression of MEK1 , EGFR and the phosphorylation of EGFR on tyrosine 1016 and 1110 between these two groups.

In the observation group, the phosphorylation of MEK1 on both serine 217 and serine 298 was significantly decreased (all $P < 0.01$) . The expression level of SPC2 in the observation group was significantly in-creased as compared with that of the control group ($P < 0.01$) . Conclusions Long-term cultivation of A549 lung cancer cells with DDW medium significantly inhibits the cell proliferation and differentiation, which is related to decreasing phos-phorylation level of MEK1, arresting the G1 /S phase transformation, inducing A549 cells from type Ⅱ alveolar epithelial cells to type I alveolar epithelial cells and improving the differentiation of A549 cells.

Key words: lung carcinoma; deuterium-depleted water; cell cycle; mitogen-activated extracellular signal-regulated kinase; cell differentiation

Deuterium is one of the three isotopes of hydrogen [1]. Naturally occurring deuterium is essential for normal cell growth [2, 3]. The natural content of deuterium varies depending on the sampling location on Earth, with a concentration range of 120 to 160 ppm [4]. Hydrogen exists most abundantly on Earth in the form of water. Water with a deuterium content of less than 150 ppm is called deuterated water (DDW). DDW can inhibit cancer cell growth in vitro and in vivo [5, 6]. It has been reported that it is used as an adjuvant treatment for patients with brain metastases from lung cancer. The use of DDW combined with conventional clinical treatment can prolong the survival of cancer patients [7]. The epidermal growth factor receptor (EGFR) and mitogen-activated extracellular signal-regulated kinase (MEK) pathways are important signaling pathways that affect tumor cell proliferation. The activation of the signaling pathway is positively correlated with the phosphorylation level of the active site [8]. From June to September 2016 , we used DDW to culture human lung adenocarcinoma A549 cells, observed the phosphorylation levels of EGFÿ and MEK1 active sites in A549 cells, and detected the expression changes of alveolar type ÿ epithelial cell-specific protein markers AQP5 and T1a and alveolar type ÿ epithelial cell-specific protein markers SPB, SPC1, SPC2, and CFTÿ to explore the effect and mechanism of DDW on the proliferation and differentiation of human lung adenocarcinoma A549 cells. 1 Materials and Methods 1.1 Materials A549 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. F12K culture medium, fetal bovine serum and 0. 25% trypsin-EDTA was purchased from Thermo Fisher, propidium iodide, BCA protein concentration assay kit and RIPA buffer were purchased from Beyotime Biotechnology Co., Ltd., CCK-8 kit was purchased from Beijing Boerma Biotechnology Co., Ltd., DDW (deuterium content 50 ppm) was purchased from Shanghai Qitian Biotechnology Co., Ltd., and antibodies for detecting total protein of MEK1 and EGFÿ, anti-MEK1 serine 217 phosphorylation (MEK-Antibodies against phosphorylated serine at position 1016 (EGFR-pSer217) and phosphorylated serine at position 217 (MEK1-pSer298) and antibodies against phosphorylated tyrosine at position 1016 (EGFR-pTyr1016) and phosphorylated tyrosine at position 1110 (EGFR-pTyr1110) were purchased from Cell Signaling, USA; antibodies against AQ5, T1a, SPC1, SPC2 and GADPH were purchased from Abcam, USA; antibodies against CFTÿ and SPB were purchased from Merk Millipore, USA; secondary antibodies coupled with horseradish peroxidase (HÿP) were purchased from KPL, USA; chemiluminescent reagents were purchased from GE, USA; and other chemical reagents were purchased from Sigma, USA. 1.2 Cell grouping and treatment A549 cells were divided into control group and observation group, and F12K medium prepared with normal water,Glutamine at a final concentration of 2 mmol/L and 10% fetal bovine serum were added to the F12K medium prepared with DDW . The cells were cultured at 37°C, 5% CO2, and saturated humidity for 30 days. The cell morphology was observed under an inverted microscope every day.

1.3 Cell proliferation observation The cells cultured for 30 days were digested with 0.25% trypsin-EDTA and inoculated in a 96-well culture plate at 1 × 105/mL. 10 ÿL of CCK-8 reagent was added at 12, 24, 36, and 48 h, respectively, and the culture was continued for 90 min to develop color. The absorbance OD value was measured at a wavelength of 450 nm using an enzyme reader. Three replicate wells were set at each time point, and the experiment was repeated 3 times, and the average value was taken. 1.4 Cell cycle analysis The cells cultured for 30 days were added with 0.25% trypsin-EDTA and inoculated in a 96-well

culture plate at 1 × 105/mL. 25% trypsin-EDTA digestion, 1 × 105 / mL inoculated into 6-well culture plates. After cell attachment, the control group and observation group cells were starved in normal water medium and DDW medium without serum for 48 h to synchronize cell growth, and then added with normal water medium and DDW medium containing 10% fetal bovine serum for 36 h and 48 h. 48 h. Trypsin was added for digestion, and the cells were resuspended in The cells were stained in propidium iodide staining solution for 30 min in the dark. FACS CaliburTM was used to analyze the DNA content in single cells The distribution of cell populations in different phases of the cell cycle was analyzed using ModFit LT software was used for fitting analysis.

1.5 Expression of EGFÿ and MEK1 proteins and detection of active site phosphorylation Western blot was used for detection.

Pre-chilled PBS containing protease inhibitors and phosphatase inhibitors Wash, add RIPA lysis buffer and repeatedly blow to lyse the cells, and centrifuge at 4 °C The supernatant was collected from the centrifuge and the total protein concentration was determined using a BCA kit. 50 ÿg of protein sample in each lane was subjected to SDS-PAGE and then wetted Transfer the protein bands to PVDF membranes. Milk powder was blocked with PBST, and the corresponding antibodies (EGFR, MEK1 antibodies) were added. 4ÿ primary antibody incubation After washing 3 times with PBST, the cells were incubated with a secondary antibody conjugated with HRP.(1ÿ10000) incubated at room temperature for 1 h, washed with PBST, and chemiluminescent substrate Colorimetric, chemiluminescent scanners to record protein expression or protein activity The target protein and the internal reference protein GADPH The relative expression level of the target protein was calculated by the ratio of gray values.

1.6 Detection of alveolar epithelial cell-specific protein markers

1. The total protein of the two groups of cells was extracted by the method in 5, and then electrophoresed and transferred to the membrane.

Western blot analysis was then performed using antibodies against AQ5, T1a, CFTÿ, Antibodies against SPB, SPC1, and SPC2 were used to detect the expression of each protein marker. Calculated by the ratio of the gray value of the target protein to the internal reference protein GADPH Relative expression of target protein. ÿ

1.7 Statistical methods were performed using SPSS 17.0 software.

± s, and one-way analysis of variance was used for comparison among groups. P ÿ 0.05 The difference was statistically significant.

2 Results

2.1 Comparison of cell morphology between the two groups Under the microscope, it is cubic, with close contact and stacked growth between cells; Observe The cells in the group were spindle-shaped, elongated, and the contact between cells was smaller than that in the control group. The group is loose.

2.2 Comparison of cell proliferation between the two groups

The OD values at 48 h were 0.33 ± 0.12, 0.38 ± 0.01, and 0.42, respectively. ± 0.02, 0.50 ± 0.06; the control group were 0.33 ± 0.02, 0.37 ± 0.01, 0.47 ± 0.05, 0.65 ± 0.05. Observation group 36, The OD value at 48 h was lower than that in the control group (all $P < 0.05$). 2.3 Comparison of cell cycle distribution between the two groups The observation group was in The proportion of cells in the G1 phase was higher than that in the control group, and the proportion of cells in the S phase The cell cycle of the two groups was significantly decreased at 48

h. There was no statistically significant difference in the proportions. See Table 1.

Table 1 Comparison of cell cycle distribution between the two groups (%, ± s)

Group	36 h			48 h		
	G1	S	G2 /M phase	G1	S phase	G2 /M phase
Observation	phase 70.51 ± 3.12*	phase 22.15 ± 0.95*	6ÿ 01 ± 0ÿ 47	57ÿ 25 ± 1ÿ 02	30ÿ 02 ± 1ÿ 49	12ÿ 86 ± 1ÿ 15
group Control group	56ÿ 69 ± 2ÿ 13	34ÿ 28 ± 1ÿ 04	6ÿ 98 ± 0ÿ 31	64ÿ 35 ± 1ÿ 97	27ÿ 16 ± 0ÿ 74	8ÿ 05 ± 0ÿ 63

Note: Compared with the control group, * P ÿ 0.01.

2.4 Comparison of MEK1 and EGFÿ phosphorylation levels between the two groups

There was no statistical difference in the expression of MEK1 protein between the control group and the Compared with the control group, the phosphate groups of serine 217 and 298 in the observation group The expression of EGFÿ protein in the observation group was significantly lower than that in the control group. There was no statistical difference; the phosphorus of tyrosine at position 1016 and 1110 There was no significant change in acidification levels.

Table 2 Phosphorylation levels of MEK1, EGFÿ and their active sites in the two groups (ÿx ± s)

Group	MEK1	MEK1 pSer217	MEK1 pSer298	EGFÿ	EGFÿ pTyr1016	EGFÿ pTyr1110
Observation	0ÿ 27 ± 0ÿ 04	0ÿ 50 ± 0ÿ 09	0ÿ 35 ± 0ÿ 06	0ÿ 52 ± 0ÿ 03	0ÿ 37 ± 0ÿ 04	0ÿ 22 ± 0ÿ 01
group Control group	0ÿ 32 ± 0ÿ 07	1ÿ 32 ± 0ÿ 21	1ÿ 09 ± 0ÿ 23	0ÿ 52 ± 0ÿ 05	0ÿ 36 ± 0ÿ 07	0ÿ 27 ± 0ÿ 08

2.5 Comparison of alveolar epithelial specific marker levels between the two groups After 30 days of culture, the levels of AQP5, T1a, SPC1, and SPC2 in the observation group were significantly higher than those in the control group. The levels of CFTR and SPB in the control group were not significantly changed. The differentiation type of some A549 cells in the observation group changed from type II epithelial cells toTransformation of type I epithelial cells. See Table 3.

3 Discussion

Table 3 Comparison of alveolar epithelial specific marker levels between the two groups (ÿx ± s)

Group	AQP5	T1a	CFTÿ	SPB	SPC1	SPC2
Observation	1ÿ 26 ± 0ÿ 11	0ÿ 69 ± 0ÿ 07	0ÿ 69 ± 0ÿ 07	0ÿ 61 ± 0ÿ 16	0ÿ 78 ± 0ÿ 07	1ÿ 15 ± 0ÿ 19
group Control group	0ÿ 54 ± 0ÿ 04	0ÿ 50 ± 0ÿ 03	0ÿ 60 ± 0ÿ 14	0ÿ 58 ± 0ÿ 17	0ÿ 43 ± 0ÿ 08	0ÿ 33 ± 0ÿ 05

Studies have shown that deuterium can affect cell growth [8].

DDW can significantly prolong the survival of patients with prostate cancer and non-

metastatic lung cancer However, DDW has a significant inhibitory effect on tumor cell growth. The molecular mechanism is still unclear. The whole process, S phase DNA replication, M phase cell division, The S phase and the M phase are preceded by the G1 growth phase and the G2 growth phase, respectivelyÿ10ÿ. This study showed that DDW could inhibit the transition of A549 cells from G1 phase to S phase. This growth inhibition effect is due to DDW blocks the cell cycle from G1 phase to G2 phase. Studies have shown that A549 cells carry the oncogene KÿAS / G12S activation mutation, this mutation upregulates the MAPK/ERK signaling pathway, thereby promoting the division and proliferation of A549 cells [11, 12]. In order to explore the signaling pathway of DDW's inhibitory effect on A549 cell growth, we selected two important molecules closely related to cell mitosis, EGFÿ and MEK1, and studied the changes in phosphorylation levels at key phosphorylation sites related to their activity. The results showed that DDW culture had no significant effect on the expression of EGF ÿ and the phosphorylation levels of its 1016th and 1110th tyrosine active sites; although it had no significant effect on the expression level of MEK1, it could significantly reduce the phosphorylation levels of its 217th and 298th serines. This indicates that DDW can inhibit the proliferation of A549 cells by downregulating the MEK/ERK signaling pathway.

Alveolar epithelium is divided into type I and type II according to its morphology and function. Type I epithelium is mainly responsible for gas exchange, while type II epithelium maintains the surface tension of alveoli by secreting surfactant proteins [9]. A549 cells belong to type II alveolar epithelial cells and can produce surfactant proteins [9, 13]. This study found that after culturing A549 cells with DDW medium for 30 days, the cells underwent significant morphological changes, from the original cubic shape, close cell-to-cell contact and stacked growth to spindle-shaped, elongated cells and less changed. Western blot further detected the expression levels of alveolar epithelial cell-specific protein markers and found that DDW can significantly upregulate the expression of two type I alveolar epithelial specific protein markers, AQP5 and T1a, while the expression of two type II epithelial specific protein markers, SPC1 and SPC2, is also significantly increased. The above changes in cell morphological phenotypes and specific marker levels indicate that DDW can induce the transformation of A549 cells from type II alveolar epithelial cells to type I alveolar epithelial cells, thereby increasing the degree of differentiation of A549 cells and decreasing their malignant proliferation ability.

In summary, DDW can block the cell cycle of A549 cells and inhibit cell proliferation by downregulating the MAPK/ERK signaling pathway; DDW can also induce the transformation of A549 cells from type II alveolar epithelium to type I alveolar epithelium. These research results partially explain the molecular mechanism of DDW inhibiting lung cancer cell proliferation and provide a basis for the use of DDW in the adjuvant treatment of lung cancer.

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