Effect of Bitter Melon (*Momordica Charantia* Linn) on Level and Function of Natural Killer Cells in Cervical Cancer Patients with Radiotherapy

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Abstract

Cervical cancer patients have a defective immune system. There is a decrease of total white blood cell count including lymphocytes and natural killer (NK) cells. NK cells, one type of lymphocytes, play a role to eliminate cancer cells by antibody dependent cell mediated cytotoxicity (ADCC) mechanism. Previous studies have shown that P-glycoprotein (170 kDa, transmembrane protein) may be a transporter for cytokine releasing in ADCC mechanism.

This study proposed to explore the role of bitter melon intake in cervical cancer patients undergoing normal treatment (radiotherapy). Subjects were divided into three groups: 1) normal control (women 35-55 years, n = 35), 2) patient control (n = 30) and 3) patient treatment (n = 30) groups. Patient control and patient treatment groups were cervical cancer patients (stage II or III) treated with radiotherapy (without or with bitter melon ingestion). Blood samples of patient control and patient treatment groups were analyzed for NK cells percentage and P-glycoprotein level. Bitter melon is a Thai herb. Previous studies have shown that bitter melon can stimulate lymphocyte activity *in vitro* and *in vivo* (mouse). The authors hope that bitter melon could stimulate the increase of NK cells percentage and P-glycoprotein level on the membrane in blood samples from cervical cancer patients who ingest bitter melon.

The results showed an increased percentage of NK cells in patient control and patient treatment groups. The increase in each group is significant ($p < 0.05$) when compared with the percentage of NK cells from second and third blood sampling time (after radiation with of without bitter melon intake for 45 and 90 days) with first blood sampling time (before treatment). The results also show a significant decrease of P-glycoprotein level ($p < 0.05$) in second and third blood sampling times when compared with first blood sampling time of the patient treatment group. There was no significant difference of P-glycoprotein (P-gp) level from first, second and third blood sampling times in patient control group.

Bitter melon ingestion did not affect NK cell level but it affected the decrease of P-gp level on NK cell membrane.

Key word : Bitter Melon, Natural Killer Cells, Cervical Cancer

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At present, cervical cancer is the most common type of cancer causing death among Thai women in northern Thailand(1). The major side effects of cervical cancer treatment are the low immunity condition of patients and anemia. Low immune condition shows the low total count of white blood cells (WBC) that also leads to a low level of lymphocytes(2). Subsets of lymphocytes consist of T cells (CD3+), B cells (CD19+), NK cells (CD56+) and NKT cells (CD3+CD56+). They play a role in cancer cell elimination as immunomodulating cells especially the natural killer cells (NK). NK cells eliminate cancer cells by the antibody dependent cell mediated cytotoxicity (ADCC) mechanism.

Previous studies found that P-glycoprotein (170 kDa transmembrane protein) is involved in the ADCC mechanism. They showed that P- gp may be a transporter for cytokines (granzyme and perforin) releasing(3-7).

Bitter melon (*Momordica charantia* Linn) is a Thai herb and previous studies have shown that bitter melon could stimulate the immune system *in vitro* and *in vivo*(8-10).

This study investigated the effect of bitter melon on NK cells level and P-glycoprotein level on NK membrane in cervical cancer patients

MATERIAL AND METHOD

Differential of subjects and collection of blood samples

The subjects were divided into 3 groups, normal control group, patient control group and patient treatment group. All the subjects were women aged 35-55 years old. The normal control group consisted of women who received a check up at Lampang regional cancer center with normal results. Patient control and patient treatment group consisted of women with cervical cancer (stage II–III) who were not pretreated in other hospitals before admission to Lampang regional cancer center.

K3EDTA blood samples were obtained three times from the patient treatment and patient control groups by venepuncture. The first time, when the patients began treatment with radiation, the second and third time, after the patients had been treated with radiation (plus bitter melon ingestion or not) for 45 and 90 days.

Bitter melon dose intake: 6 capsules/day; each capsule contained bitter melon powder 300 mg/ capsule and the patient treatment group ingested 2 capsules after meals three times a day for 90 days.

Peripheral Blood Mononuclear Cell (PBMC) preparation from blood samples by ficoll–hypaque gradient centrifugation technique(3,11)

PBMCS were prepared by mixing K_3EDTA blood samples with phosphate buffer saline (FBS) in a proportion 1 : 1 by volume, underlay with ficoll-hypaqne in the proportion 1 : 2, then centrifuged at 400 g for 30 min. The PBMC layer was separated and washed twice by centrifuged at 1,000 g 5 min. Then, cells were adjusted to the desired concentration in PBS.

Natural Killer (NK) cell counts and analysis of P-glycoprotein level on cell membrane

PBMCS were resuspended in 1 per cent BSA-PBS-azide and mixed with AB serum at 4°C for 30 minutes. PBMCs were then stained with 20 µl of monoclonal antibodies (anti-IgG1/2, anti-CD3*FITC/CD16/56*PE) anti-P-gp*FITC and anti-CD45* FITC/CD14*PE). Cells were incubated at 4°C in the dark for 30 minutes and washed twice with 1 per cent BSA-PBS-azide. Cells were fixed with 1 per cent paraformaldehyde. The number of NK cells was counted and P-gp level was analyzed by a FACsort flow cytometer(3,12,13).

All of the results were statistically analyzed by student *t*-test (*t*-test two-sample assuming equal variance).

RESULTS

Fig. 1 summarizes the percentage of each lymphocyte subset. The percentage of NK cells, T cells, B cells, NKT cells were as follows: 18.04 ± 8.63, 58.10 ± 8.75, 19.69 ± 5.70 and 4.17 ± 3.36 respectively. The reference range percentage of the Thai population was 7.28 ± 41.71, 50.31 ± 80.58, 7.73 ± 25.35. No reference value of NKT cells was reported(14).

P-gp was expressed on the membrane of all lymphocyte subsets but at different levels. This was related to antibody dependent cells-mediated cytotoxicity mechanism at the level of granzyme and perforin releasing(3-7). Among mononuclear cells that express functional P-gp, it is interesting to speculate as to the physiologic role for this membrane transporter. P-gp in leucocytes could function to transport cytokines, cytotoxic effect molecules or inflammatory mediators. In the present study, the P-gp levels were determined by flow cytometry in terms of Mean Fluorescence Intensity (MFI).

P-gp levels on NK cell membrane were examined by measuring the intensity of the fluorescent substance that was conjugated with an anti-P-gp antibody. Fig. 2 shows the MFI of NK cells, B cells and lymphocytes at 28.88 ± 9.98, 18.53 ± 6.53 and 22.87 ± 11.93 respectively. These results show that NK cells expressed the highest level of P-gp among all lymphocyte subsets. P-gp level on monocyte membranes (33.5 ± 11.68) was higher than P-gp level on lymphocyte membranes (22.87 ± 11.93).

To analyze the effect of bitter melon intake as a modulator of NK cells and P-gp, the authors determined the percentage of cell population and the level of P-gp in blood samples obtained from patient control and patient treatment groups before and after bitter melon ingestion. When the percentage of NK cells from blood samples was compared beginning at day 0, there was no difference between the two groups (Fig. 3). The percentage of NK cells from the second blood sample of patient control and patient treatment

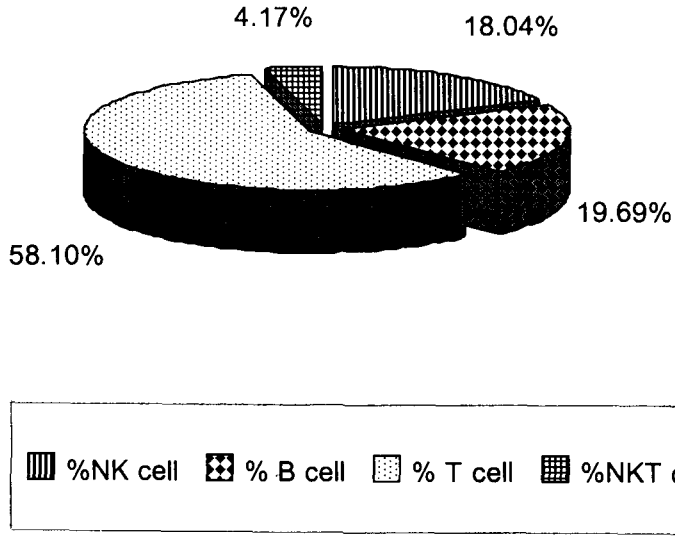

Fig. 1. The percentage of lymphocyte subsets in the normal control group analyzed by flow cytometry (n = 35).

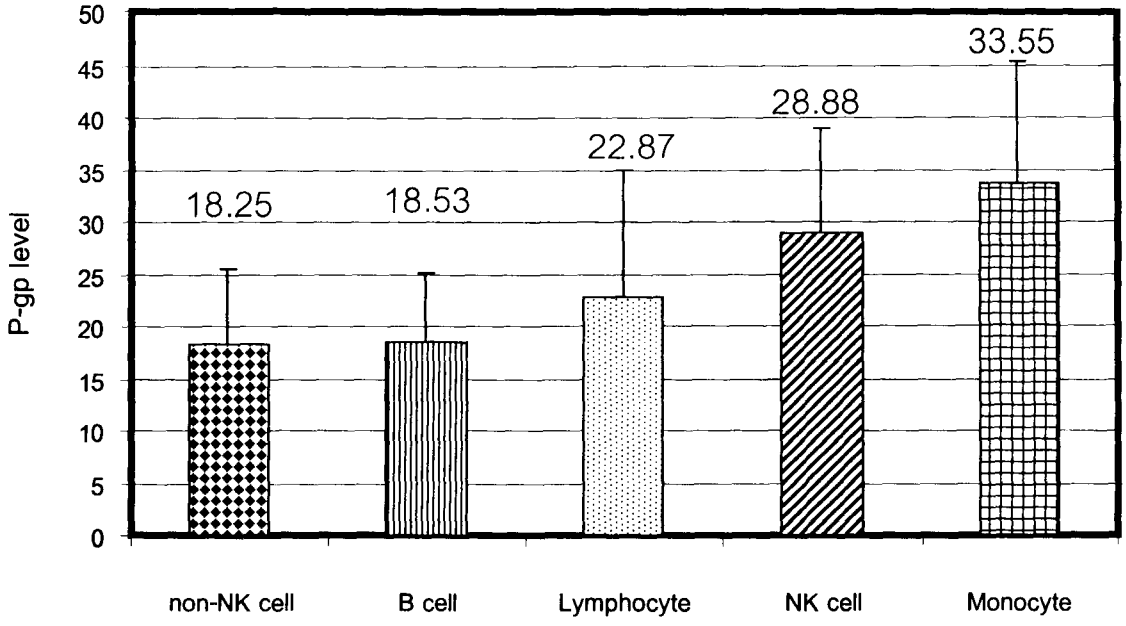

Fig. 2. The Mean Fluorescence Intensity (MFI) of P-gp positive cells in the normal control group analyzed by flow cytometry. Data are displayed as mean values of 35 patients.

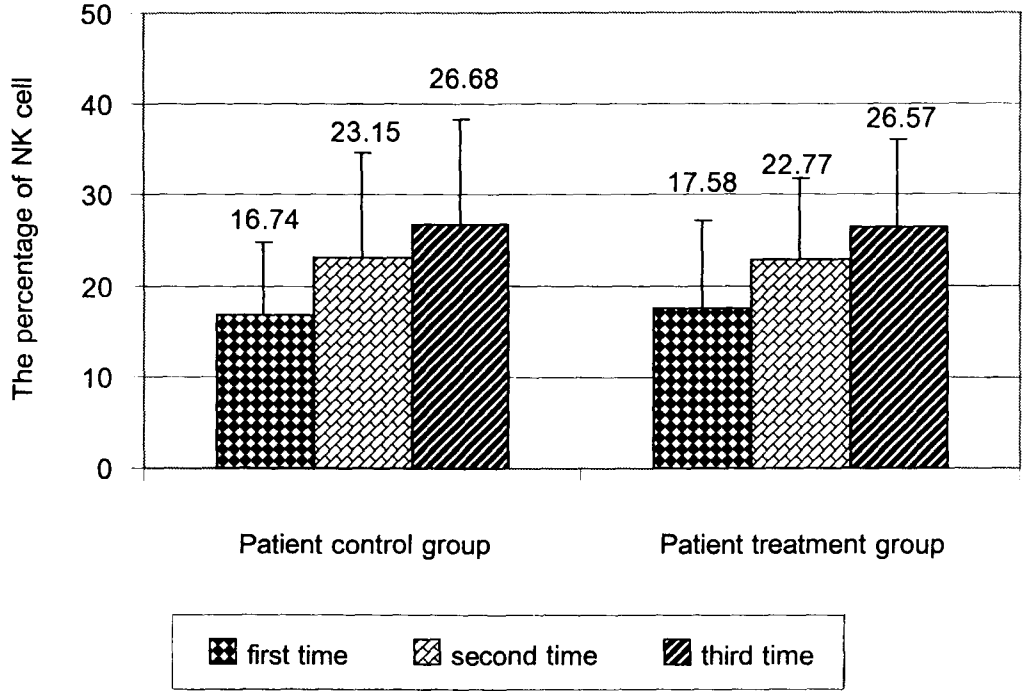

Fig. 3. The percentage of NK cells from 1st, 2nd and 3rd blood sampling times in patient control and patient treatment groups analyzed by flow cytometry. Data are displayed as mean value of 30 patients per group.

groups was significantly higher than the first ($p = 0.01$ and $p = 0.03$ respectively). The percentage of NK cells from the third blood samples of the two groups was also significantly higher than the first time ($p = 0.01$ and $p = 0.0007$ respectively). To analyze the effect of bitter melon on the percentage of NK cells, the authors compared the mean difference of the percentages by three ways : second time and first time (2^{nd} - 1^{st}) third time and first time (3^{rd} - 1^{st}), third time and second time (3^{rd} - 2^{nd}) in the patient control group and patient treatment group. The results showed no change in the mean difference of NK cell per cent from any of the three sorts in either group. From these results, it was concluded that bitter melon intake did not effect the percentage of NK cells in cervical cancer patients.

The P- gp level on NK cells is shown in Fig. 4. In the patient control group, there was no significant difference of P-gp levels between first, second and third blood sampling times (day 0, day 45 and day 90). In the patient treatment group, however, the P-gp level on NK membrane was decreased significantly when comparing the second and first time ($p = 0.0007$), third and first time ($p = 0.01$). When comparing the third and second time, there was no significant difference. In the patient treatment group, the bitter melon was given along with radiotherapy. These results indicated that bitter melon intake may cause a decrease in P-gp level on the NK cell membrane.

DISCUSSION AND SUMMARY

Cervical cancer patients' health depends on their immune system. Natural Killer cells (NK cells), immunomodulating cells play an important role in cancer cell elimination[15,16]. NK cells eliminate cancer cells by antibody dependent cell-mediated cytotoxicity (ADCC)[15,16]. Thus, a high proliferation of these cells could help the prognosis of the patients. P-gp, a transmembrane protein on the NK cell membrane, correlates with the ADCC mechanism[6,17-20]. It acts as a transporter of cytokine releasing. NK cells with high level of P-gp could have better benefit than low expression cells[6,17-22].

Bitter melon is a Thai herb. Several studies have reported the effect of bitter melon on cancer cells. Crude extract from bitter melon killed human leukemic lymphocytes while not affecting the viability of normal human lymphocyte cells at the same dose[23]. Fruit and seeds of bitter melon could activate murine lymphocytes for anti-leukemic and antiviral function[10]. Crude extract of bitter melon could inhibit mouse skin papillomagenesis[9] and inhibit

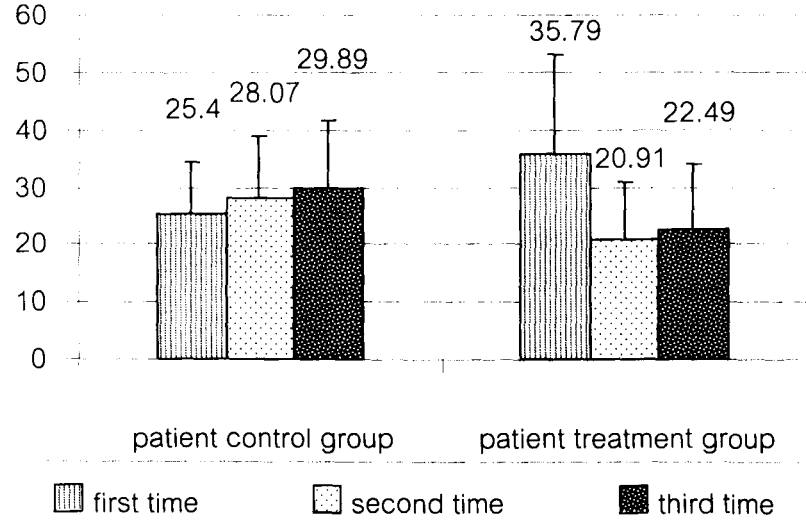

Fig. 4. The P-gp level of NK cells from 1^{st}, 2^{nd} and 3^{rd} blood sampling times in patient control and patient treatment groups analyzed by flow cytometry. Data are displayed as mean value of 30 patients per group.

tumor formation in CBA/H mice which had been given i.p. injection of CBA/DI tumor cells[8]. From these reports, the authors studied the effect of bitter melon on lymphocyte especially NK cells in cervical cancer patients. Lymphocyte is an immunomodulating cell and divides into several sub-populations including T cells, B cells, NK cells and NKT cells. Each type of cell expresses different CD molecules on its membrane. Lymphocyte sub-populations can be separated by immunophenotyping using specific antibodies to CD molecules[14]. T cells express CD3 antigen, whereas, B cells do not express CD3 antigen but express CD19 molecules. NKT cells express CD3, CD16 and CD56 antigen while NK cells express CD16 and CD56 but not express CD3. Thus, in the present study, anti-CD3 conjugated with FITC and anti-CD16 plus CD56 conjugated with PE were used to identify each subset of lymphocytes. The expression of P-gp on lymphocyte sub-populations was also determined by the immunofluorescent technique. The techniques used in this study, both determining lymphocytes and P-gp expression, were the same as those reported by Chaudhary et al[20] and Walter TK[12].

In the present study, the authors analyzed the number of lymphocyte sub-populations including T cells, B cells, NK cells in healthy volunteers and cervical cancer patients and analyzed the P-gp level on NK cell. It was found that the percentage of NK, B and T cells of the healthy volunteer group was in the same range as the reported Thai healthy volunteers[14]. When comparing the percentage of NK cells in cervical cancer patients and healthy volunteers, it was found that the percentage of NK cells in cancer patients was lower than the normal control group; however, it was not statistically significant.

Comparing before and after treatment with radiation, the results showed an increase of NK cell percentage after treatment with radiation 45 days and 90 days in patients both with and without giving bitter melon. This increase was statistically significant.

The effect of bitter melon intake on the number of NK cells in cervical cancer patients after treatment with radiation was examined. From the results of the percentage of each cell type, it was concluded that the level of NK cells in cervical cancer patients was lower than the normal control group but not significant. The NK cell level increased after treatment with radiation, and bitter melon did not affect the level of NK cells. Because NK cells are essential for cervical cancer patients, it was anticipated that the immune system of patients judge to increase the NK cells level.

In the present study, the function of NK cells was analyzed by detecting the level of P-gp on their membrane. The results showed a decrease of P-gp level on NK cell membrane of patients who ingested bitter melon, whereas, there was no change of P-gp level on NK cell membranes of patients who were treated only with radiation. The decrease of P-gp levels resulted in impaired NK cell function.

From the results, it was concluded that bitter melon ingestion did not affect the NK cell level. Bitter melon ingestion reduced the P-gp level on NK cell membranes and may cause the impairment of immunomodulating cells. From these conclusions, the authors suggest that bitter melon ingestion is not beneficial for cervical cancer patient when treated with radiation. However, bitter melon did reduce the multi-drug resistant phenomenon and so it may be useful for patients being treated with chemotherapy.

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ผลของมะระขี้นกที่มีต่อจำนวนและหน้าที่ของ Natural killer (NK) cells ในผู้ป่วยมะเร็งปากมดลูกร่วมกับการรักษาด้วยการฉายรังสี

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ผู้ป่วยโรคมะเร็งปากมดลูกมีภาวะภูมิคุ้มกันต่ำ พบว่าจำนวนเม็ดเลือดขาวทั้งหมดรวมถึงเซลล์เม็ดเลือดขาวชนิดลิมโฟไซท์และเซลล์เอ็นเค จะมีจำนวนลดลงด้วย เซลล์เอ็นเคเป็นเซลล์เม็ดเลือดขาวลิมโฟไซท์ชนิดหนึ่งซึ่งมีหน้าที่ในการกำจัดเซลล์มะเร็งโดยขบวนการ antibody dependent cell mediated cytotoxicity (ADCC) จากผลการวิจัยที่ผ่านมาพบว่า พี-ไกลโคโปรตีน (โปรตีนที่แทรกตัวอยู่บนผนังเซลล์ มีน้ำหนักโมเลกุล 170 กิโลดาลตัน) มีความเกี่ยวข้องกับขบวนการ ADCC โดยพบว่า พีไกลโคโปรตีนอาจจะเป็นช่องทางสำหรับการหลั่งของสาร cytokines ต่าง ๆ ได้แก่ granzyme และ perforin ออกมาจากเซลล์เอ็นเค

การศึกษาในครั้งนี้ต้องการศึกษาถึงผลของการให้ผู้ป่วยโรคมะเร็งปากมดลูกรับประทานมะระขี้นกควบคู่ไปกับการรักษาด้วยการฉายรังสี โดยแบ่งกลุ่มที่ต้องการศึกษาออกเป็น 3 กลุ่ม คือ กลุ่มคนปกติ (ผู้หญิงที่มีอายุระหว่าง 35-55 ปี จำนวน 35 คน) กลุ่มผู้ป่วยควบคุมและกลุ่มผู้ป่วยทดลอง ซึ่งเป็นผู้ป่วยโรคมะเร็งปากมดลูก ระยะที่ 2 หรือ 3 อายุระหว่าง 35-55 ปี กลุ่มละ 30 คน กลุ่มผู้ป่วยควบคุมจะได้รับการฉายรังสีเพียงอย่างเดียว ในขณะที่กลุ่มผู้ป่วยทดลองจะได้รับประทานมะระขี้นกควบคู่ไปกับการฉายรังสี ตัวอย่างเลือดที่เก็บจากทั้ง 3 กลุ่ม จะนำมาตรวจวัดจำนวนเซลล์เอ็นเค และวัดระดับพี-ไกลโคโปรตีนโดยวิธีโฟไซโตเมทรี

มะระขี้นกเป็นสมุนไพรไทย จากการศึกษาที่ผ่านมาพบว่า มะระขี้นกสามารถกระตุ้นการทำงานของเม็ดเลือดขาวลิมโฟไซท์ทั้งในการทดลองแบบ *in vitro* และ *in vivo* (หนู) ผู้วิจัยคาดว่ามะระขี้นกจะสามารถกระตุ้นให้เกิดการเพิ่มจำนวนเซลล์เอ็นเคและระดับพีไกลโคโปรตีนบนผิวเซลล์ในผู้ป่วยที่ได้รับประทานมะระขี้นกควบคู่ไปการการฉายรังสี

ผลการทดลอง พบว่าจำนวนของเซลล์เอ็นเคเพิ่มขึ้นในกลุ่มผู้ป่วยทั้ง 2 กลุ่ม หลังจากได้รับการรักษา ทั้งที่ไม่ได้รับประทานและรับประทานมะระขี้นก การเพิ่มขึ้นของเซลล์เอ็นเคหลังการรักษาในแต่ละกลุ่ม เป็นการเพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.05$) และพบว่าเมื่อนำตัวอย่างเลือดก่อนเข้ารับการรักษามาตรวจวัดเทียบกับตัวอย่างเลือดหลังการรักษา ในกลุ่มผู้ป่วยที่ได้รับประทานมะระขี้นก พบว่าระดับของพีไกลโคโปรตีนลดลงหลังได้รับการรักษาอย่างมีนัยสำคัญ แต่ผู้ป่วยกลุ่มที่ได้รับการฉายรังสีเพียงอย่างเดียว พบว่าระดับพีไกลโคโปรตีนในตัวอย่างเลือดก่อนและหลังได้รับการรักษาไม่มีความแตกต่างกัน จึงสรุปได้ว่า มะระขี้นกไม่มีผลต่อการเพิ่มจำนวนของเซลล์เอ็นเค แต่มีผลทำให้มีระดับพีไกลโคโปรตีนบนผิวเซลล์เอ็นเคลดลง

คำสำคัญ : มะระขี้นก, มะเร็งปากมดลูก

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