Intense nanosecond pulsed electric fields promote cancer cell apoptosis through centrosome-dependent pathway involving reduced level of PLK1

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Abstract. – BACKGROUND: Intense nanosecond pulsed electric fields (nsPEFs) have been known to promote apoptosis without physically changing membrane structure or damaging morphology of tumor cells. To determine the contribution of centrosome to the progression of apoptosis by nsPEFs, HeLa cells were exposed to high intensity (6 kV/cm) nsPEFs (8-32 ns) in normal culture condition and cell biology and molecular parameters of cells were investigated.

MATERIALS AND METHODS: Apoptotic cell death was identified by TUNEL assay after being exposed to the nsPEFs with various pulse durations, while immunofluorescent staining was performed to detect the number and distribution of centrosomes. To clarify whether nsPEFs-induced centrosome over-duplication is the consequence of DNA damage, we used comet assay to detect simultaneous DNA damage. And additionally Western Blot was used to detect PLK1 protein level to explore the correlation between apoptotic cell death and nsPEFs-induced centrosome over-duplication. Correlation between nsPEFs and molecular parameters was statistically analyzed.

RESULTS: NsPEFs induced a clear apoptosis reaching a maximum at 24ns, 24h after exposure (p < 0.05), where DNA fragmentation and over-duplicated centrosomes were observed. This apoptosis may be promoted in a time- and pulse duration-dependent manner. Polo-like kinase (PLK1) protein levels were significantly decreased by such nsPEFs (p < 0.05). Control treatment without the nsPEFs did not cause any damage to the cultured HeLa cells.

CONCLUSIONS: Intense nsPEFs promote cell apoptosis through a centrosome-mediated pathway involving a reduction in the level of PLK1, which may provide new therapeutic targets for human cancer treatment.

Key Words: Nanosecond pulsed electric fields, Centrosome, DNA fragmentation, Apoptosis, PLK1, Hela cells.

Introduction

The effect of intense pulsed electric fields (PEFs) on biological cells and tissues has been extensively studied since the late 1950’s. Neumann and Rosenheck firstly reported in 1972 permeability changes induced by PEFs of membrane, which was termed as electroporation. This effect has usually been used for transdermal drug delivery, clinical electrochemotherapy, facilitation of gene transfection without viral vectors, and the development of nucleic acid vaccines. Typical pulses for electroporation range from tens of milliseconds with intensity of a few hundreds V/cm² to a few microseconds and several kV/cm². Recently, the pulse duration range has been shortened into the nanosecond range along the improvement of equipment. With increasing intensity and decreasing duration of PEFs, the effects of such short pulses have been revealed to induce intracellular responses and apoptosis in the absence of membrane permeability changes and other structural effects associated with long duration electroporation. Typical pulses for intracellular effects are as brief as several or tens of nanoseconds with high amplitudes. Apoptosis is an energy-dependent series of reactions through inherent intracellular signaling cascades that proceed to the systematic disassembly.
of the cell without disrupting the function of or provoking responses from the adjacent cells. Aberrant apoptosis is an important feature in tumorigenesis, and induction of tumor cell apoptosis is one major aim of chemotherapy and radiotherapy due to its excellent advantages, i.e. causing tumor cell death without local inflammation and pain, and also scarring of the surrounding normal cells.

In most animal cells, centrosome is an intracellular organelle that serves as the main microtubule organizing center (MTOC) and a regulator of cell-cycle progression. Abnormal centrosome number is a common phenomena in tumors and usually occurs after DNA damage or fragmentation. In normal cells, accurate cell division requires DNA to be replicated without error and the replicated chromosomes to be segregated equally between the two newly formed daughter cells. Thus centrosome duplication is closely correlated with the cell cycle and centrosome number is tightly linked to the DNA content of a cell. It is reported that the formation of overduplicated centrosomes can lead to mitotic abnormalities, such as the formation of multipolar spindles, which could, in turn, result in abnormal chromosome segregation and aneu-ploidy. Given the clear consequences of centrosomal abnormalities for the cell, such as causing apoptosis, it is important to investigate the molecular pathways underlying those centrosomal abnormalities and the consequences of those abnormalities in cancer cells. Such information may be used to develop novel and effective therapeutic ways to cause cancer suppression and restriction. Centrosome, an intracellular organelle, is linked closely to DNA fragmentation, and may be directly or indirectly affected by the nsPEFs.

One of the principal signalling molecules involved in instigating apoptosis after DNA damage and centrosome overduplication is Polo-like kinase 1 (PLK1). PLK1 has been demonstrated to function as a key regulator of mitotic events by phosphorylating substrate proteins on centrosomes, kinetochores. More importantly, PLK1 is necessary for the functional maturation of centrosomes for the establishment of a bipolar spindle. It is also reported that depletion of PLK1 can induce apoptosis in cancer cells.

So far, there is no evidence indicating the effect of nsPEFs to evoke apoptosis associated with centrosome abnormality. This reported study is designed to investigate the specific effect of nsPEFs on centrosome and PLK1, as well as the apoptosis of tumor cells in vitro.

**Materials and Methods**

**Cell Culture**

Human cervical cancer cell line HeLa cells were used in this experiment. This cell line was obtained from Gynecological Cancer Laboratory of West China Second Hospital, Sichuan University. The cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640) (GIBCO, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (GIBCO), 100 ug/ml streptomycin and 100 Units/ml penicillin (GIBCO) at 37°C in a humidified incubator with 5% CO2.

**Nanosecond Pulse Generators and Corresponding Exposure Control**

The energy-controllable nanosecond pulse generator was invented by the Institute of Applied Physics, University of Electronic Science and Technology of China. It generated nanosecond pulse electric fields, and the output pulse wave was in the form of exponential attenuation with very short rising time. The peak voltage was adjusted to 6 kV, the pulse duration was 8-32 ns, and the frequency was 10 Hz. In nsPEFs experiment, the electrodes were inserted into culture solution for 1 min total stimulations, the space between two electrodes was set at 1 cm. Exposure controls were performed with same electrodes for same period of 1 min without electrical stimulation.

**TUNEL Assay**

Apoptotic cell death was identified by TUNEL System (Cat. 11684795910, Roche, Mannheim, Germany) according to the manufacture’s protocol. Then the cells were counterstained with 1 µg/mL 4,6-diamidino-2-phenylindole (DAPI, Cat. 10236276001, Roche, Mannheim, Germany) for 10 min to identify all the nucleated cells. HeLa cells were cultured in Petri-dishes (8.5 cm²). After the culture period, cells were re-suspended and exposed to the nsPEFs with various pulse durations of 8, 16, 24, 32 ns for one min. The control group was having electrodes inserted into culture medium for the same period but without applying nsPEFs. In relation to the nsPEFs exposure, TUNEL staining was performed at 0h, 1h, 4h, 12h, 24h, 48h, or 72h and observed under a fluorescence microscope respectively. The total number of apoptotic cells in 50 randomly selected cells in 5 randomly selected fields was counted. The apoptotic ratio was
calculated as the percentage of positive staining cells among total counted cells, namely Ratio = number of apoptotic cells/50 total examined cells.

**Centrosome Labeling**

As TUNEL assay, HeLa cells were re-suspended before the nsPEFs. After exposure, the cells were transferred into 24-well chamber with slides in it. Based on the TUNEL results, the cells were treated in the pulse durations of 24 ns nsPEFs for one min. After the exposure, centrosome staining was detected at 8h and 24h time points respectively. This time, however, the cells were reattached to slides already, and washed twice with cold phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at 4°C. After blocked with 5% BSA/PBS, the cells were incubated overnight at 4°C with primary antibody as rabbit polyclonal anti-human centrin antibody at a dilution of 1:200 (Cat. ab11257; Abcam, Cambridge, UK). Following overnight incubation with the first antibody, fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Cat. ZF-0311; Zhongshan Goldenbridge Biotechnology, China) were applied to the slides and incubated at 37 C for 1 h followed by several washes of PBS. Then slides were treated with 1 µg/mL DAPI (4',6-diamidino-2-phenylindole) for 10 min, and washed again, Images were acquired and analyzed with the fluorescence microscope (Nikon Eclipse Ti). The exposure time, amplifier gain and offset values were controlled by the NIS-Elements AR 3.0 software and were held constant throughout the experiment.

**Comet Assay**

DNA damage was detected and analyzed quantitatively by comet assay 19 one hour after cells exposed to the nsPEFs. In this procedure, 100 µL 1% LMP agarose was dripped onto a slide and placed at 4°C for 10 minutes to be solidified. The cell suspension was mixed with 1% low-melting-point (LMP) agarose (Amresco, Solon, OH, USA) at 37°C (1:10 volume per volume), and 80 µL of this mixture was layered onto the chilled agarose. The slide was placed at 4 C for another 10 min. Then, a final layer of 1% LMP agarose was added on top of slides. The slide was immersed in pre-chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, and 1% Triton X-100), at 4°C for 2 h, and then washed three times with PBS. The slide was then transferred into electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min to untwist the DNA, and electrophoresis was performed (25 V, 300 mA for 20 min). The slide was then placed in neutralization solution (0.4 M Tris, pH 7.5) for three periods of 5 min. DNA was stained by dropping 5 µg/mL DAPI onto the slide and, after 15 min, slides were inspected under a fluorescent microscope (Nikon Eclipse Ti Nagao dai Machi, Ti, Japan). The length of the comet tail (in pixels) was calibrated using the software Image-Pro Plus 6.0 (Bethesda, MD, USA). Fifty cells in 5 visual fields were sampled randomly for assay in each group.

**Western Blot Analysis**

HeLa cells were cultured in Petri-dishes (8.5 cm²) and re-suspended before exposed to nsPEFs, then harvested after 1h, 2h, 4h and 8h after stimulation by nsPEFs for 1 min (24 ns). Total protein was extracted by Cell Lysis Buffer (RIPA, cocktail and Phenylmethanesulfonyl fluoride, Beyotime Institute of Biotechnology, China), following manufacturer’s instructions. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C, and the supernatants were stored at −80°C. Protein concentration was determined using the BCA assay kit (Cat. PA002, SinoBio Biotech, Shanghai, China). A 50 µg protein of from each cellsample was mixed with loading buffer (100 mM Tris, pH 6.8; 200 mM dithiothreitol (DTT); 4% sodium dodecyl sulfate (SDS); 20% glycerol; 0.2% bromophenol blue) at 1:1 dilution, boiled for 5 min, then loaded on to 12% SDS-PAGE gel for electrophoresis and transferred to a pure nitrocellulose blotting membrane (Millipore Corporation, Billerica, MA, USA). The loaded membranes were blocked for 1 h in 5% defatted dry milk/TBS-T (Tris Buffered Saline-Tween at room temperature. Subsequently, the membranes were incubated with anti-PLK1 antibody (diluted 1:2000, Cat. ab47867, Abcam, Cambridge, UK) and anti-β-actin antibody (diluted 1:1000, Cat. TA-09, Zhongshan Goldenbridge Biotechnology, China) in TBS-T containing 5% defatted milk overnight at 4 C respectively. After being washed three times in TBS-T, the membranes were incubated for 1 h in 5% defatted milk/TBS-T containing a 1:5,000 dilution of HRP (Horseradish Peroxidase)-conjugated goat anti-rabbit for PLK1 or goat anti-mouse for β-actin antibodies (Cat. ZB-2301/2305, Zhongshan Goldenbridge Biotech-
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Technology, China). Blotted proteins were visualized and analyzed with the ECL (enhanced chemiluminescence) system (CWBiotech, Beijing, China) according to the manufacturer’s instructions.

**Statistical Analysis**

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze multiple comparisons. Post-hoc analysis was used for paired data. A statistically significant difference was defined as \( p < 0.05 \).

**Results**

**The nsPEFs Promote Apoptosis in HeLa Cells**

To determine whether the nanosecond pulse generators induce cell apoptosis, we performed cell apoptosis assay using TUNEL method. We found that at 0 h just before the nsPEFs exposure, almost no apoptotic cells were detected. At 1 h after exposure, apoptosis began to be detected and was significantly increased over time to reach peak level at about 24 h after stimulation (24 ns group TUNEL positive %: 0±0, 20±2.4, 35±1.8, 78±9.6, 95±8.2, 50±6.6, 30±5.0 at 0h, 1h, 4h, 12h, 24h, 48h, 72h, respectively, \( p < 0.05 \)). From 24 h, late apoptosis or necrosis increased gradually. Meanwhile, the apoptotic cell percentage of each experimental group with different pulse durations was higher than the corresponding control one, and pulse duration for 24 ns has the highest percentage of all (Figure 1, 24h after nsPEFs, TUNEL positive %: 4±0.8, 80±10.2, 88±6.0, 96±7.4, 76±9.2 in control, 8 ns, 16 ns, 24 ns, 32 ns group respectively, ANOVA, \( p < 0.05 \)). These results suggested that nsPEFs might promote apoptosis of HeLa cells in a time- and pulse duration-dependent manner.

![Figure 1](image.png)

*Figure 1.* Comparison of HeLa cells before and after treatment by 24 nsPEFs on TUNEL fluorescent staining (200×HPF; apoptotic cells were detected as bright green intensity located in whole cell). TUNEL analysis showed that after 24 nsPEFs treatment, the apoptotic cells were statistically increased compared to the control group without nsPEFs (*\( p < 0.05 \)).
The nsPEFs Promote Over-Duplication of Centrosomes in HeLa cells

It has been evidenced that nsPEFs would affect intracellular organelles and induce apoptosis\(^7\). Also, it is reported that over-duplication of centrosomes could initiate cell death\(^20\). Therefore, we further investigated whether the nsPEFs in this experimental system exerted an influence on centrosome to cause apoptosis in HeLa cells. The results shown in Figure 2 confirmed this prediction. Through 24 ns nsPEFs exposure, the number of centrosomes in single cell and percentage of cells with abnormal supernumerary centrosomes were significantly increased 8h after the exposure. At 24h after the exposure, the phenomenon remained but weakened, suggesting a start of repairing process, which turned certain percentage of cells to normal centrosome numbers. The total number of centrosomes in 50 randomly selected cells from 5 randomly selected fields (10 each field) was counted, and the percentage of cells with more than double-centrosomes was calculated under microscope at 400× amplification. Both the average number of centrosomes in each cell and the percentage of cells with more than double-centrosomes increased significantly in nsPEFs groups compared to control group (Table I, \(p < 0.05\)).

<table>
<thead>
<tr>
<th>The number of centrosomes in every single cell</th>
<th>The percentage of cells with centrosomes (\geq 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group (24ns)</td>
<td>4.8(^*)</td>
</tr>
<tr>
<td>Control group</td>
<td>1.6(^*)</td>
</tr>
</tbody>
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\(t\)-test: *\(p < 0.5\).
The nsPEFs Promote DNA Damage in HeLa Cells

It was previously implicated that the over-duplication of centrosomes was induced by DNA damage\(^\text{14}\). To clarify whether nsPEFs-induced centrosome over-duplication is the consequence of DNA damage, we used comet assay to detect simultaneous DNA damage. In this assay, DNA damage is assessed by single-cell gel electrophoresis, which leads to the migration of broken DNA out of the cell body giving rise to a tail of DNA fragments. Unbroken DNA remains in the cell nucleus as a head of the comet\(^\text{21}\). In this experiment, cells were treated with nsPEFs with same parameters as these used to cause over-duplication of centrosomes and scored by fluorescence microscopy for the distribution of DNA between the “tail” and the “head”\(^\text{21}\). Cells with increased tail to head ratio of fluorescence signals were defined as DNA damaged cells. We compared the percentage of DNA damaged cells as well as the length of comet tail between the nsPEFs treated group and control group. The total number of DNA damaged cells in 50 randomly selected cells from 5 randomly selected fields was counted. As shown in Figure 3, after nsPEFs, DNA damaged cells were significantly increased from 5.8% to 91.2% (\(p < 0.05\)), and the length of comet tails were much longer than those in control group (30.8 \(\mu\)m to 143.7 \(\mu\)m, \(p < 0.05\)). These results show a similar proportional change of DNA damage and over-duplication of centrosomes by same nsPEFs treatment.

Polo-Like Kinase 1 (PLK1) Protein Level was Down Regulated After nsPEFs

Recent observations have shown that inhibition of PLK1 results in induction of apoptosis and spindle formation in some cancer cells\(^\text{13}\). To determine whether nsPEFs-induced apoptosis is coincided with PLK1 depletion, we further investigated PLK1 protein levels after the nsPEFs treatment. Same parameters of nsPEFs treatment were used to mimic the experimental conditions.

![Figure 3](image-url)

**Figure 3.** 24 nsPEFs treatment induced DNA damage. **(a)** Comet assay in HeLa cells with or without nsPEFs treatment. After 24 nsPEFs treatment, tail fluorescence signal was bigger and more intense. In addition, the comet tails were much longer and cells with increased tail to head ratio of fluorescence signals (*\(p < 0.05\)). **(b)** Comparison of DNA damaged cells between nsPEFs treated and control groups. **(c)** Comparison of comet tail length between nsPEFs treated and control groups.
causing DNA damage and over-duplication of centrosomes. The PLK1 protein level was reduced significantly by the nsPEFs treatment. In addition, to clarify the tendency of protein level changes over variable time period, we selected five time points after the nsPEFs treatment as 1h, 2h, 4h, 8h and 16h respectively. These results showed that at the time points of 1h, 2h and 4h, the PLK1 protein expressions were only slightly reduced without significant difference. At 8h after exposure, the PLK1 protein expression was significantly reduced (Figure 4), suggesting that the down-regulation of PLK1 protein level after nsPEFs exposure may be time-dependent.

**Discussion**

Recently, malignant tumor therapy has moved to the comprehensive treatment mode with multiple approaches simultaneously. With the development of electrical and biomedical engineering technology, the permeabilization of the plasma membrane of biological cells by external applied electric fields has been used extensively in biotechnology and medical areas, such as chemotherapy combined by membrane perforation caused by electric field pulses. This effect is evoked by PEFs with a pulse duration of longer than 10 s, as under such condition the cell membrane acts as a capacitor and has to be charged to a sufficient voltage to cause membrane defects in the electrical field without selection. Lately, it has been reported with short pulse durations on the order of ns for the charging time of the plasma membrane, increasing electrical field causes effects on intracellular components and consequently the induction of tumor cells apoptosis without unspecific membrane perforation. It is well known that abnormal or reduced cell apoptosis is one of the possible causes of cancer. Disorder of apoptosis is closely related with the dysfunction of abnormal cells clearance in the early process of cancers. It is hypothesized that disorder of apoptosis in cancer cells may play a role in the cancer development, and that nsPEFs may be used to treat tumor by inducing apoptosis of cancer cells with certain specificity. It is shown here that high intensity nsPEFs cause a significant cancer apoptosis in this report.

This current report also demonstrates that intracellular centrosomes were affected, which may result in apoptosis in HeLa cells after exposing to nsPEFs. Interestingly, it was demonstrated previously that nsPEF invokes signal transduction mechanisms, which initiate apoptosis cascades in several other human cancer cell lines including HL-60 cells and Jurkat cells with electric fields as high as 300 kV/cm. However, in the study reported here, we found that the nsPEF with relatively lower electrical fields (6 kV/cm) could trigger apoptosis signal similar to those with intensive high electric fields in HeLa cell lines. Our findings suggest that the same therapeutic effect can be reached with lower fields, and consequently with lower thermal effect and lighter damage to the surrounding tissues; which are very important in considering the safety of nsPEFs as a cancer treatment method. Cancer type selectivity will need to be investigated further in the future to set-up possible guild for therapeutic use of nsPEFs clinically.

In the present study, we performed several pulse durations from 8 to 32 ns and detected apoptosis at different time point after nsPEFs treatment. It has been reported that shorter pulses, which have lesser plasma membrane effects, result in slower apoptosis progression, whereas longer pulses, which have greater plasma membrane effects, result in more rapid apoptosis progression. Our data, however, do not show the same tendency. All four duration groups from 8 to 32 ns caused a maximal apoptosis ratio 24h after nsPEFs. Besides, we found that there was no regularity in apoptosis ratio in relation to the pulse duration. Unexpectedly, the pulse duration of 24 ns showed the most pronounced effect on cell apoptosis. Although the actual mechanisms
underlying the phenomenon are still unclear, we infer that this disparity may be caused by the cell type specificity. Different cell types may have different organelle sizes and distributions. Considering a cell as a conductor surrounded by a loss, insulating envelope, and containing substructures with similar properties, various cell types may differ markedly\(^2\). Nevertheless, the detailed molecular biological mechanism underlying apoptosis caused by nsPEFs needs to be investigated thoroughly.

In view that nsPEFs have been reported mainly to affect intracellular organelles, we speculated that nsPEF may have effects on the cell interior that activates inherent pathways to cause apoptosis. Stacey et al\(^{24}\) once reported that nsPEF applications resulted in increased levels of DNA damage and cell death, and increased perturbations in cell cycle parameters in cells. However, the normal cell division requires the replicated chromosomes, which then attach to microtubules emanating from the opposite poles of a bipolar mitotic spindle and congress to a metaphase plate. Upon anaphase initiation, the sister chromatids disjoin and move to the spindle poles. Therefore, if this process was disturbed, cells may divide abnormally and result in cell death. Based on this hypothesis, current experiment demonstrated that the number of centrosomes in cells was increased significantly and also the distributions were much more dispersed after nsPEFs, i.e. the number of cells with over-duplication of centrosomes increased significantly by nsPEFs. As previous study has indicated that DNA damage could induce centrosome duplication\(^{25}\), DNA damage-induced centrosome duplication may serve as a mechanism for ensuring death of cells that evade the DNA damage or spindle assembly checkpoints. The reported over-duplication of centrosomes in tumor cell after treated by nsPEFs may also be caused by DNA damage.

It is progressively accepted that multiple centrosomes can cause nucleate microtubules and contribute to the assembly of multipolar spindles, and, thereby, segregating the chromosomes into more than two fragments. Cells containing multiple nuclear fragments are not able to survive because of the irreparable chromosomal damage\(^{26}\). The form of cell death has been generally classified as either apoptosis or mitotic cell death, because these two processes may be linked in many cases\(^{28}\). Sato et al\(^{26}\) also indicated that it was nuclear damage resulted from multiple centrosomes as the trigger of apoptosis. Based on these previous findings, we demonstrated that nsPEFs-induced apoptosis of tumor cells did contain centrosome over-duplication.

Nevertheless, the precise mechanism by which DNA damage leads to abnormal number of centrosomes is not yet clear. Here we examined the apoptosis-related cell cycle-specific protein (Polo-like kinase 1, PLK1), which localized on centrosome during mitosis before anaphase, and moved into the kinetochore/centromere during anaphase. It is, therefore, suggested that PLK1 may regulate chromosome and chromatin separation. PLK1 is necessary for the functional maturation of centrosome in late G2/early prophase and for the establishment of a bipolar spindle. Inhibition of PLK1 leads to apoptosis in both direct and indirect pathways\(^{13}\). In fact, depletion of PLK1 causes a blockade of Cdc25C activation, which serves as a checkpoint for DNA damage during G2 preventing activation of cyclin B/Cdc2 complex. As a consequence, G2 phase arrest is induced. In cells arrested for an abnormally long time in G2, the centrosome cycle might begin again ever though mitosis has not been traversed to cause over-duplication\(^{26, 27}\). Present study demonstrated that PLK1 protein expression was significantly reduced after nsPEFs, suggesting that the DNA damage-reduced PLK1 pathway may be involved in the over-duplication of centrosomes after nsPEFs, and subsequent apoptosis directly or indirectly.

**Conclusions**

Present study provides a possible mechanism of cancer apoptosis caused by nsPEFs as centrosome-dependent. The over-duplication of centrosomes in cancer cells after treated by nsPEFs may be a consequence of DNA damage-induced reduction in PLK1 pathway. Such information may be useful to identify a new therapeutic target with novel treatment methods for human cancer treatment.

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References


