Research Article

$^{125}$I Seeds Radiation Induces Paraptosis-Like Cell Death via PI3K/AKT Signaling Pathway in HCT116 Cells

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$^{125}$I seeds brachytherapy implantation has been extensively performed in unresectable and rerecurrent rectal carcinoma. Many studies on the cancer-killing activity of $^{125}$I seeds radiation mainly focused on its ability to trigger apoptosis, which is the most well-known and dominant type of cell death induced by radiation. However, our results showed some unique morphological features such as cell swelling, cytoplasmic vacuolation, and plasma membrane integrity, which is obviously different from apoptosis. In this study, clonogenic proliferation was carried out to assay survival fraction. Transmission electron microscopy was used to analyze ultrastructural and evaluate morphologic feature of HCT116 cells after exposure to $^{125}$I seeds radiation. Immunofluorescence analysis was used to detect the origin of cytoplasmic vacuoles. Flow cytometry analysis was employed to detect the size and granularity of HCT116 cells. Western blot was performed to measure the protein level of AIP1, caspase-3, AKT, p-Akt (Thr308), p-Akt (Ser473), and $\beta$-actin. We found that $^{125}$I seeds radiation activated PI3K/AKT signaling pathway and could trigger paraptosis-like cell death. Moreover, inhibitor of PI3K/AKT signaling pathway could inhibit paraptosis-like cell death induced by $^{125}$I seeds radiation. Our data suggest that $^{125}$I seeds radiation can induce paraptosis-like cell death via PI3K/AKT signaling pathway.

1. Introduction

Colorectal cancer remains one of the most common causes of cancer-related deaths worldwide [1]. There were 715,000 patients who died from colorectal cancer in 2010 [2]. Colorectal cancer is more common in developed countries than developing countries [3]. The main causes of colorectal cancer are overweight, changes in dietary patterns, and physical inactivity [4]. The local recurrence rate of colorectal cancer is up to 21–46% [5]. Although primary radical resection and postoperative external beam radiotherapy have been widely carried out in pelvic recurrence patients, the therapeutic effect is poor due to the serious complication and poor prognosis [6]. And local recurrence has become the greatest barrier in the treatment of colorectal cancer.

As a salvage treatment, $^{125}$I seeds brachytherapy implantation is feasible, effective, and safe for patients with unresectable and rerecurrent rectal carcinoma [7]. To date, most studies have demonstrated that $^{125}$I seeds radiation exerts cancer-killing activity by suppressing the metastasis of tumors or triggering apoptosis [6, 8]. The work presented here characterizes a novel form of cell death in response to $^{125}$I seeds radiation. We found besides apoptosis that $^{125}$I seed radiation killed colorectal cancer cell via inducing paraptosis. Paraptosis induced by several natural products such as coelomocyte extracts, honokiol, gamma-Tocotrienol, curcumin, and berberine in anticancer treatment receives more and more attention in recent years [9–13]. Paraptosis is a kind of caspase-independent programmed cell death and is characterized by distinct cytoplasmic vacuolization derived from swelling endoplasmic reticulum and/or mitochondria. This form of cell death is fundamentally different from apoptosis and lacks some distinct characteristics of apoptosis such as DNA fragmentation, pyknosis, or caspase activation and cleavage [14]. Moreover, the expression of AIP1 is specifically inhibited in paraptosis cells, while it is not affected in apoptotic cells [15]. Paraptosis lacks typical necrotic morphology such as plasma membrane blebbing.
And paraptosis is also insensitive to apoptotic and autophagic inhibitor [10].

However, the mechanisms underlying paraptosis have not yet been fully understood. Curcumin-induced paraptosis has been reported to be positively associated with ERK2 and JNK (c-jun N-terminal kinase-1) activation [16]. In addition, insulin-like growth factor I receptor- (IGFIR-) induced paraptosis has been reported to be inhibited by MEK-2-specific inhibitors and by antisense oligonucleotides directed against JNK-1 [15]. We further focused our interest on the molecular mechanisms that underlie 125I seeds radiation-induced paraptosis on colorectal cancer cells. We found that PI3K/AKT signaling pathway involved the modulation of 125I seeds radiation-induced paraptosis.

2. Materials and Methods

2.1. Radiation Source. 125I seeds which have a half-life of 59.4 days were obtained from Ningbo Junan Pharmaceutical Technology Company (Ningbo, Zhe Jiang province, China). The activity of 125I seeds was 2.5 mCi and the initial dose rate was 2.77 cGy/h. The 125I seeds were installed in an in-house 125I seeds radiation model described in detail in the previous published paper [8, 17]. The exposure time for delivering radiation doses of 0.5, 1, and 2 Gy was 17.69, 35.54, and 71.71 hours, respectively.

2.2. Antibodies and Materials. The primary antibodies against Akt, p-Akt (Thr308), Calnexin, and p-Akt (Ser473) were purchased from CST (Cell Signaling Technology). LC3 and TIM23 antibody were obtained from Sigma-Aldrich. Goat anti-rabbit IgG-horseradish peroxidase (HRP), goat anti-mouse IgG-HRP, and anti-β-actin mAb were purchased from Sigma-Aldrich. Anti-AIP1 antibody was obtained from Santa Cruz. Cycloheximide was obtained from MedChem Express (Princeton, NJ, USA).

2.3. Cell Culture. HCT116 cells were kindly provided by professor Xiaojuan Du of the health center of Peking University. The cells were passaged when the cells reach 80% confluency. The cells were cultured in 1640 medium with 10% fetal bovine serum supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin in an atmosphere containing 5% CO2 at 37°C.

2.4. Surviving Fractions. HCT116 cells in appropriate number were seeded in 35 mm dishes to make sure of the formation of about 100 colonies per dish. After cell adhesion, the cells were exposed to 2 Gy of 125I seeds radiation in the model. After removal from radiation source cells were kept culturing for 10–12 days. Cells were fixed with 4% formaldehyde in PBS and stained with methylene blue. The number of colonies containing 50 cells per dish was counted to calculate survival fraction. The survival fraction (SF) was defined as the ratio: PE (irradiated group)/PE (unirradiated group), where PE was (colony number/number of inoculating cells) × 100% [18]. The experiment was repeated three times in duplicate.

2.5. Cell Viability Assay. Following irradiation HCT116 cells were digested with trypsin and stained with trypan blue dye for 5 min at room temperature. Cell viability was determined by trypan blue exclusion.

2.6. Transmission Electron Microscopy (TEM). After exposure to 2 Gy of 125I seeds radiation HCT116 cells were fixed with 2.5% glutaraldehyde in PBS. After postfixing with 1% osmium tetroxide for two hours, the samples were dehydrated in a graded series of ethanol solutions. The samples were cut followed by embedding in epoxy resin. The ultrathin sections (70 nm) were loaded on copper slot grids after staining with uranyl acetate and lead citrate. The presentative images were captured using a JEM1400 transmission electron microscope.

2.7. Immunofluorescence. HCT116 cells were seeded on cover-slips of 35 mm dishes. After exposure to 125I seeds radiation cells were fixed with 4% formaldehyde in PBS and permeabлизized with 0.2% Triton X-100 in PBS for 15 minutes on ice. Subsequently, the cells were blocked with goat serum for 30 minutes and incubated with primary antibodies overnight at 4°C. ER and mitochondria were detected with the anti-Calnexin and anti-TIM23 primary antibody, which were labeled with corresponding FITC (Fluorescein Isothiocyanate) (green) and TRITC- (Tetramethyl Rhodamine Isothiocyanate-) conjugated secondary antibody (red). Nucleus was stained with DAPI (4’,6-diamidino-2-phenylindole) (Beyotime, China). Fluorescence images were obtained using LSM 510 Zeiss confocal microscope (Carl Zeiss Jena, Germany).

2.8. Western Blotting. Following irradiation HCT116 cells were harvested and washed with cold PBS. The cell precipitates were lysed with RIPA lysis buffer on ice for 15 minutes and centrifuged at 12000 g at 4°C for 30 minutes. BCA kit was used to measure protein concentration of supernatant. Protein was denatured at 95°C for 5 minutes and equal amount of sample (50 µg of protein per sample) was loaded on SDS-PAGE gel. The protein was separated by 10–12% SDS-PAGE gel and transferred onto PVDF membranes (Hybond-p, Amersham, Buckinghamshire, UK), which were blocked with 5% nonfat milk in TBST (1% Tween-20 in 20 mM TBS, pH 7.6). The membranes were incubated with specific primary antibodies at 4°C overnight and then washed with PBS three times for 5 minutes. The membranes were incubated with the following corresponding horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. ECL kit (Santa Cruz) was used to visualize protein bands.

2.9. Statistical Analysis. Results were presented as mean ± SD (standard deviation). Statistical analysis was performed using SPSS (17.0) software. The differences between means of groups were determined by Student’s t-test. p < 0.05 was considered significant.

3. Results

3.1. Effects of 125I Seeds Radiation on Growth of HCT116 Cells. Following irradiation survival fraction of HCT116 cells after exposure to 2 Gy of 125I seeds radiation was shown in Figure I(a). Data showed that human colorectal cancer cells were
sensitive to 2 Gy of $^{125}$I seeds radiation. After irradiation, only 0.1% of the HCT116 cells remained clonogenic. Moreover 25% of HCT116 cells were dead and did not exclude trypan blue following irradiation (Figure 1(b)). As shown in Figure 1(c), flow cytometry analysis showed that $^{125}$I seeds radiation caused the increase of cell volume and cell granularity. Microscopic image showed that the control group cells grew close together, whereas the HCT116 cells exposed to $^{125}$I seeds radiation loosed tight contact amongst cells. Furthermore, a large number of cytoplasmic vacuoles appeared in HCT116 cells exposed to $^{125}$I seeds radiation (Figures 1(d) and 1(e)). This result was consistent with the LS180 cells treated with $^{125}$I seeds radiation which has been published previously [8]. After removal from $^{125}$I seeds radiation cytoplasmic vacuoles persisted with progression of time. And the size and numbers of vacuoles even increased over time (24–48 h) (Figure 2).

3.2 $^{125}$I Seeds Radiation Induced Paraptosis-Like Cell Death. To investigate morphological changes induced by $^{125}$I seeds radiation, following irradiation HCT116 cells were analyzed by transmission electron microscopy. After treatment of 2 Gy of $^{125}$I seeds radiation, numerous rounding and large vacuoles

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**Figure 1**: The effects of $^{125}$I seeds radiation on HCT116 cells. HCT116 cells were harvested after exposure to 2 Gy of $^{125}$I seeds radiation. (a) Clonogenic survival fraction was assessed. (b) Cell viability of irradiated cells was quantified by trypan blue exclusion. (c) The size and granularity of HCT116 cells were evaluated by flow cytometry. (d) The morphological changes of HCT116 cells were visualized using inverted microscopy (magnification: $\times$40). (e) The percentage of vacuolar cell was quantified. The values are the means $\pm$ SD. *$p < 0.05$.
Figure 2: Cytoplasmic vacuolation induced by $^{125}\text{I}$ seeds radiation is nonreversible. The morphological changes of HCT116 cells were observed by inverted microscope (magnification: ×40) with progression of time (24–48 h) after removal of $^{125}\text{I}$ seeds radiation.

Figure 3: $^{125}\text{I}$ seeds radiation induced the morphological changes of HCT116 cells. (a) 48 h after exposure to 2 Gy of $^{125}\text{I}$ seeds radiation the morphology of HCT116 cells was detected under transmission electron microscopy. Control cell with normal mitochondria and ER, which was denoted by white arrows or black arrowheads, respectively. Irradiated cells showed cytoplasmic vacuolation. Mitochondria were denoted by white arrowheads. (b) DAPI staining of HCT116 cells 48 h after irradiation.

3.3. Cytoplasmic Vacuoles Induced by $^{125}\text{I}$ Seeds Radiation Were ER-Origin. To further investigate the origin of vacuoles, immunofluorescence staining with anti-TIM23 and anti-Calnexin antibody was performed. As shown in Figure 4(a), the cytoplasmic vacuoles induced by $^{125}\text{I}$ seeds radiation colocalized with ER detected by Calnexin antibody. Moreover, cytoplasmic vacuoles did not colocalize with mitochondria stained with TIM23 antibody after exposure to $^{125}\text{I}$ seeds radiation, which rule out the involvement of mitochondria in the origin of the vacuoles. To examine whether the vacuoles are autophagosomes, LC3 (microtubule associated light chain protein), a specific autophagosome marker, was examined by immunofluorescence analysis. The punctation staining of LC3 appeared after exposure to $^{125}\text{I}$ seeds radiation. However, these punctuations did not localize inside the vacuoles; these results suggested that these vacuoles were not of autophagosome-origin (Figure 4(b)).

3.4. The Expression of AIP1 Decreased after Exposure to $^{125}\text{I}$ Seeds Radiation. Since the inhibition of AIP-1 is the specific marker for paraptosis [15], to investigate whether the expression of AIP1 decreased after exposure to $^{125}\text{I}$ seeds radiation, western blotting was performed to assess the expression of AIP1. As shown in Figure 5, the expression of AIP1 declined compared to control group following gradually increased dose of $^{125}\text{I}$ seeds radiation. In addition, following irradiation, the protein expression of caspase 3 and cleaved caspase 3 did not increase compared to β-actin.

3.5. Cycloheximide (CHX) Reversed $^{125}\text{I}$ Seeds Radiation-Induced Cytoplasmic Vacuolization. As we know, protein synthesis is required in paraptotic process [19]. To evaluate the effect of CHX on $^{125}\text{I}$ seeds radiation-induced cytoplasmic vacuolization, we pretreated HCT116 cells with 20 µM of CHX, an eukaryote protein synthesis inhibitor, for 2 h before exposure to $^{125}\text{I}$ seeds radiation. The results showed

appeared in HCT116 cells, which is different from character of apoptosis such as cell shrinkage, apoptosis body, or chromatin condensation (Figure 3(a)). Moreover, DAPI staining showed that the nucleus was intact after exposure to 2 Gy of $^{125}\text{I}$ seeds radiation (Figure 3(b)).

AIP1 decreased after exposure to $^{125}\text{I}$ seeds radiation, western blotting was performed to assess the expression of AIP1. As shown in Figure 5, the expression of AIP1 declined compared to control group following gradually increased dose of $^{125}\text{I}$ seeds radiation. In addition, following irradiation, the protein expression of caspase 3 and cleaved caspase 3 did not increase compared to β-actin.

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that CHX protects HCT116 cells from $^{125}$I seeds radiation-induced cytoplasmic vacuolization, which suggested that $^{125}$I seeds radiation-induced cytoplasmic vacuolization requires protein synthesis (Figure 6).

3.6. $^{125}$I Seeds Radiation Activated PI3K/AKT Signaling Pathway. Increased kinase activation is a common event in cancer cells after exposure to radiation [20–23]. We thus tested whether $^{125}$I seeds radiation affected the protein expression of PI3K/AKT signaling pathway. Western blotting was used to detect the expression of AKT, p-Akt (Thr308), and p-Akt (Ser473). Western blotting analysis showed that p-Akt (Thr308) and p-Akt (Ser473) increased gradually after exposure to increasing radiation dose of $^{125}$I seeds radiation (Figure 7), indicating that PI3K/AKT signaling pathway was activated by $^{125}$I seeds radiation.

3.7. Ly294002 Inhibited the Activation of PI3K/AKT Signaling Pathway Induced by $^{125}$I Seeds Radiation. To detect whether Ly294002, a specific inhibitor of PI3K/AKT signaling pathway, inhibited the activation of PI3K/AKT signaling pathway induced by $^{125}$I seeds radiation, we performed western blotting to analyze the expression of AKT, p-Akt (Thr308), and p-Akt (Ser473). As shown in Figure 8, Ly294002 inhibited the phosphorylation of AKT. These results showed that Ly294002...
Figure 5: After exposure to 2 Gy of $^{125}$I seeds radiation the expression of AIP1 decreased. Cells lysates were harvested 48 h after exposure to the 0 Gy, 0.5 Gy, 1 Gy, and 2 Gy of $^{125}$I seeds radiation. The protein expressions of AIP1 and caspase 3 were determined by western blotting. (a) Equal amounts of cell lysates from control and irradiated cells were analyzed. The expressions of AIP1 and caspase 3 were analyzed by western analysis with anti-AIP1 and anti-caspase 3 antibody. $\beta$-Actin was evaluated as a loading control. (b)–(d) The expression of AIP1 (b), caspase 3 (c), and cleaved caspase 3 (d) was quantified using Photoshop software. The data are normalized to the $\beta$-actin and presented as means ± SD, $^*p < 0.05$.

Figure 6: Cycloheximide reversed $^{125}$I seeds radiation-induced cell death and cytoplasmic vacuolization. HCT116 cells were pretreated with 20 $\mu$M of CHX for 2 h before exposure to 2 Gy of $^{125}$I seeds radiation. The morphological changes of HCT116 cells were showed under inverted microscope 48 h after irradiation.

inhibited the activation of PI3K/AKT signaling pathway induced by $^{125}$I seeds radiation.

3.8. Ly294002 Inhibited the Paraptosis Induced by $^{125}$I Seeds Radiation. To investigate whether Ly294002 inhibited the paraptosis induced by $^{125}$I seeds radiation, light microscopic images showed that Ly294002 decreased the percentage of vacuole in HCT116 cell after exposure to 2 Gy of $^{125}$I seeds radiation (Figures 9(a) and 9(b)). To investigate the functional significance of AKT activation in the anticancer effect of $^{125}$I seeds radiation, cell viability assay was performed to assess HCT116 cells pretreated with and without Ly294002 (10 $\mu$M) 2 hours prior to being exposed to 2 Gy $^{125}$I seeds radiation. Following irradiation the percentage of dead HCT116 cells of Ly294002 and control group was 0.74 and 0.58. There was significant difference in cell viability between Ly294002
Figure 7: $^{125}\text{I}$ seeds radiation activated PI3K/AKT signaling pathway. Cells were harvested 48 h after exposure to the gradually increased dose of $^{125}\text{I}$ seeds radiation. The expressions of AKT, p-Akt (Thr308), and p-Akt (Ser473) were determined by western blotting analysis. (a) Equal amounts of cell lysates from control and irradiated cells were analyzed. The expressions of AKT and p-Akt (Thr308 and Ser473) were analyzed by western analysis with corresponding antibody. $\beta$-Actin was evaluated as a loading control. (b)-(c) The intensity levels of the protein bands of p-Akt (Thr308) (b) and p-Akt (Ser473) (c) were quantified using Photoshop software. The data are normalized to AKT and presented as means ± SD, *$p < 0.05$.

4. Discussion

According to distinct morphological features and biochemical characteristics cell death can be classified into different types of cell death. Paraptosis has several unique morphological characteristics such as plasma membrane and nucleus integrity and swelling mitochondria or ER [10, 24]. However, due to the lack of specific biomarkers and poor understanding of its biochemical mechanisms, paraptosis is often concealed by the effects of apoptosis or autophagy and thus its occurrence and distribution may be underestimated. To date, a series of studies reported that several natural products exert anticancer effects by inducing cell cycle arrest and apoptosis [6, 8, 26, 27]. However, to date, the contribution of apoptosis to treatment success is highly debated for solid tumors [28], because the response of tumor to radiotherapy is the accumulative effect of multiple kinds of cell death to identify and a novel form of cell death induced by $^{125}\text{I}$ seeds radiation may prove valuable for increasing the curative effect of $^{125}\text{I}$ seeds radiation treatment of colorectal cancers.

We focused our interest on the effects of $^{125}\text{I}$ seeds radiation on colorectal cancer cells, we found that $^{125}\text{I}$ seeds radiation induced the death of HCT116 cells and reduced the clonogenicity of cells (Figures 1(a) and 1(b)). Moreover $^{125}\text{I}$ seeds radiation caused the increase of cell volume and granularity (Figure 1(c)). After exposure to 2 Gy of $^{125}\text{I}$ seeds radiation HCT116 cells showed some unique morphological characteristics of paraptosis such as characteristic cytoplasmic vacuole (Figure 1(d)), which was consistent with the typical morphology change as described previously [6]. And the size and numbers of vacuole increased after removal from...
125I seeds radiation with progression of time (24–48 h), which indicated that the process is irreversible (Figure 2). The number and size of vacuoles increase over time. Eventually, the vacuole sizes reach a critical point of no return and the vacuolar cell cannot be revived. Rupture of paraptotic cells leads to release the immune-stimulating “danger signal” such as heat shock proteins (HSP), the high mobility group B-1 (HMGB1), and additional proteases. Paraptosis becomes a popular mechanism of cellular demise due to its involvement in initiation of the immune system, which leads to a more powerful antitumor immunity ability compared to apoptotic cell death lack of immune-stimulating capacities in the efficacy of cancer cell clearance [29, 30].

125I seeds radiation able to trigger such a form of cell death could be more promising cancer therapeutic treatment.

TEM analysis further verified that 125I seeds radiation induced cytoplasmic vacuolation in HCT116 cells (Figure 3(a)). DAPI staining showed that the nucleus is intact after exposure to 125I seeds radiation (Figure 3(b)). Immunofluorescence analysis showed cytoplasmic vacuoles originating from swelling ER instead of mitochondria or autophagosome (Figure 4). Given that ER enlargement was just one characteristic of paraptosis, we examined whether 125I seeds radiation-induced cell death in colorectal cancer cells shared other features of paraptosis. AIP1 was reported to play a prominent role in paraptosis. Inhibition of AIP1 is specific marker for paraptosis. We further detected the expression of AIP1 and found the expression of AIP1 to decline after exposure to 125I seeds radiation. Paraptosis is a kind of caspase-independent programmed cell death. We found that 125I seeds radiation did not affect the protein expression of caspase-3 in HCT116 (Figure 5). Because that protein synthesis was required in paraptosis, we examined the effect of cycloheximide on the cytoplasmic vacuolization induced by 125I seeds radiation. We found cycloheximide reversed 125I seeds radiation-induced cytoplasmic vacuolization (Figure 6). These unique morphological characteristics and molecular marker of paraptosis suggested that 125I seeds radiation induced paraptosis-like cell death.

Although paraptosis appears to differ from that of apoptosis and necrosis, we do not yet fully understand the molecular basis of paraptosis. Some protein kinases such as p38 MAPK, MEK, and JNK have been reported to participate in paraptosis [15, 31]. AKT is a kind of protein kinase involved in modulating cell growth and differentiation. The regulatory effect of AKT on paraptosis has not been reported. Our study showed that AKT was activated after exposure to gradually increased 125I seeds radiation (Figure 7). The expression
of phosphorylated AKT (Thr308, Ser473) was upregulated compared to internal control. Ly294002, a specific inhibitor of PI3K/AKT signaling pathway, is a remarkable growth-inhibitory and has apoptosis-inducing effect in many cancer cell lines, with decreased expression of phosphorylated AKT [32]. Our results showed that Ly294002 downregulated the expression of phosphorylated AKT induced by $^{125}$I seeds radiation (Figure 8). Moreover, Ly294002 inhibited cytoplasmic vacuolation induced by $^{125}$I seeds radiation which suggested the involvement of PI3K/AKT signaling pathway in $^{125}$I seeds radiation-induced paraptosis in HCT116 cells (Figures 9(a) and 9(b)). However, Ly294002 promoted cell death of HCT116 cells after exposure to $^{125}$I seeds radiation (Figure 9(c)). There are more and more evidences verifying that paraptosis-like cell death often exists in parallel with apoptosis and is controlled through activation of different signaling pathway [33]. As we know, Ly294002 (10 $\mu$M) which inhibits the paraptosis induced by $^{125}$I seeds radiation can also promote apoptosis, data not shown. The molecular mechanisms involved in the switch between paraptosis and apoptosis deserve further exploration.

In conclusion, in the present study, we found that besides apoptosis $^{125}$I seeds radiation could also induce paraptosis simultaneously in HCT116 cells. Importantly, the paraptosis induced by $^{125}$I seeds radiation showed some specific characteristics. The potency of $^{125}$I seeds to induce paraptosis and apoptosis at the same radiation dose might be based on its capability to activate several pathways such as activation
PI3K/AKT signaling pathway. Though more work have to be done to elucidate the complicated signal cascades induced by $^{125}$I seeds radiation, what we had discovered provided new evidence for the study of paraptosis as well as the interaction between different types of cell death induced by $^{125}$I seeds radiation.

**Competing Interests**

All authors of the paper have no financial and personal relationships with other people or organizations that could inappropriately influence this work.

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