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The expression of “growth arrest and DNA damage inducible genes 45 and 153” is related to apoptotic induction of cells. GADD45 is an effector gene of the tumor suppressor p53, and GADD153 is associated with cellular function of cancer prevention. Curcumin, isolated from the plant Curcuma longa (Linn.), has been investigated as a promising cancer preventive in food because curcumin, a phenolic and coloring compound, is widely ingested in the Indian subcontinent. However, the exact mechanisms of action of curcumin have not yet been clearly elucidated. Based on our successful results with green tea catechins as cancer preventive, we studied the relationship between the expression of GADD45 and 153 and apoptotic induction in human lung cancer cell line PC-9. In our study curcumin increased the expression of GADD45 and 153 in a p53-independent manner. Curcumin also inhibited the growth of PC-9 cells and induced G1/S arrest of the cell-cycle followed by strong induction of apoptosis. Treatment with GADD45 and 153 small interfering RNAs (siRNAs) inhibited the apoptotic induction in PC-9 cells by curcumin. Moreover, curcumin induced the expression of cyclin dependent kinase inhibitor genes p21 and p27, while it inhibited the expression of numerous genes, including Bcl-2, cyclin D1, CDK2, CDK4, and CDK6. All the results with PC-9 cells suggest that the up-regulation of GADD45 and 153 by curcumin is a prime mechanism in the anticancer activity of curcumin.

Key words curcumin; apoptosis; cell-cycle; lung cancer; cancer prevention.

The expression of “growth arrest and DNA damage inducible genes 45 and 153” (GADD45 and 153) is induced by both genotoxic and non-genotoxic stress.13 Although GADD45 is the first stress-inducible gene activated by p53 tumor suppressor—it can also be induced in a p53-independent manner14—the exact function of GADD45 is not yet clear. Numerous investigators have reported that GADD45 protein is a new molecular target for human cancer therapy, since it is associated with cell-cycle regulation, apoptosis, DNA repair and genomic stability, along with immune responses.15 The expression of GADD153 has been extensively studied in cells treated with various stress conditions—oxidative stress, endoplasmic reticulum stress and deprivation of essential nutrients, such as glucose and glutamine—and also cells treated with DNA damaging agents.16 Results suggest that GADD45 and GADD153 proteins play vital roles in apoptotic induction of cells, so we focused on them for our study of lung cancer prevention in humans.

Lung cancer is the leading cause of cancer-related death worldwide. In 2006 it caused 260000 deaths in Japan and an estimated 160000 deaths in U.S.A. in 2009.4,5 In Japan, lung cancer is the leading cause of death from cancer among both men and women, and death from lung cancer has been increasing by a factor of 10.7 for men and by a factor of 9.6 for women over the past 40 years.6,7 To combat this, lung cancer prevention trials were conducted in western countries in the 1990s, but they were not successful for various reasons.8 In the developing nation of Bangladesh, lung cancer for males shows the highest mortality rate because the risk of lung cancer development is increased by widespread cigarette smoking, and the 5 year survival rate is only 15%.9 The reason for the high mortality rate is that the majority of lung cancers are diagnosed in the late stages, when conventional therapeutic regimens are no longer effective.10 So the best strategy is to minimize the development of lung cancer using cancer preventive agents, and in countries like Bangladesh there is strong interest in cancer prevention with widely investigated natural products, such as green tea, curcumin, resveratrol and capsaicin. In the Indian subcontinent, curcumin, for example, is commonly ingested in daily meals with turmeric.

Curcumin is a compound found in turmeric, which is a product of the plant Curcuma longa (Linn) and contains 2–5% total spices. Since curcumin is traditionally well-known to have therapeutic effects on various types of diseases, the cancer preventive activity of curcumin is being intensively studied all over the world, and experiments with curcumin in animal models indicate it is a preventive agent against various types of cancer.11 Specifically, curcumin inhibited cell growth of various cancer cell lines, and induced apoptosis of cancer cells, such as A549.12–17 Moreover, curcumin was effective on cell-cycle regulation of the cells.18–20 Based on these results, we think that it is possible to intensify the study of the mechanisms of cancer preventive and anti-cancer activities of curcumin for lung cancer prevention in humans.

In eukaryotic cells, DNA duplication and cell division occur through well-organized regulation of the cell-cycle, which is variously regulated by cyclins, cyclin-dependent kinases and their inhibitors. Recently, numerous reports have focused on evidence that the cancer preventive activity is induced by changing the phases of cell-cycle, associated with the alteration of their regulatory components.21,22 Here, we report for the first time that curcumin strongly induced growth inhibition and apoptosis mediated through the expres-
sion of GADD45 and 153 but independent of p53, in human lung cancer cell lines PC-9 and A549. Our study on treatment with GADD45 and 153 small interfering RNAs (siRNAs) strongly indicated that the two proteins induced by curcumin are involved in the mechanisms of cancer prevention.

MATERIALS AND METHODS

Chemicals and Reagents Curcumin, 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). GADD45, GADD153 and actin antibodies were purchased from Santa Cruz Biotechnology, Inc., CA, U.S.A.

Cell Culture Human non-small-cell lung carcinoma cell line PC-9 was obtained from National Cancer Research Institute, Tokyo, Japan and A549 was purchased from American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, kanamycin, sodium bicarbonate, and L-glutamine in 5% CO2 incubator at 37 °C.

Determination of Cell Growth Inhibition PC-9 cells (4×10^5/ml) were treated with various concentrations of curcumin or vehicle for 24 and 48 h. The inhibition of cell growth was determined by trypan blue dye exclusion test. The results were based on at least three independent experiments performed in duplicate.

Cell-Cycle Analysis by Flow Cytometry PC-9 cells were treated with various concentrations of curcumin for 24 h, washed twice with phosphate buffered saline (PBS) and then fixed in ice-cold 70% ethanol overnight at −20 °C. Next, cells were resuspended in PBS containing RNase (250 μg/ml) and incubated for 30 min at 37 °C. Cells were then treated with PI (50 μg/ml) solution, incubated for 30 min in the dark, and cell-cycle was analyzed by flow cytometry (Beckman Coulter Epics ALTRA, FL, U.S.A.).

Determination of Apoptosis by Two Methods

1) The proportion of apoptotic cells was determined by DAPI staining, as reported previously. In brief, PC-9 cells were treated with various concentrations of curcumin for 48 h, fixed in ice-cold methanol for 10 min and then stained with the DAPI reagent (1 μg/ml). The percentage of apoptotic cells was calculated using fluorescence microscopy (Bioerevo, BZ900, Keyence, Japan) with at least 200 randomly selected cells. Results are based on at least three independent experiments.

2) Apoptosis was further quantified by Annexin V-fluorescein isothiocyanate (FITC) labeled apoptosis detection kit (Beckman Coulter), according to the manufacturer’s instruction. PC-9 cells were treated with various concentrations of curcumin for 48 h and the cell suspensions were washed with ice-cold PBS. Cell pellets were resuspended in ice cold binding buffer (1×10^6 cells/ml). The suspension was treated with Annexin V-FITC and PI solution, and incubated in dark for 10 min. The cell preparations were analyzed by flow cytometry repeated at least twice.

Expression of Cell-Cycle and Apoptosis Related Genes by Quantitative Real-Time Polymerase Chain Reaction (PCR) Cells were treated with various concentrations of curcumin for 24 h. Total RNA was isolated by RNAiso reagent (Takara, Japan), and then subjected to reverse-transcription, as described previously. Expression levels of genes, including GADD45, GADD153, p21, p27, cyclin D1, Bcl-2, Bax, CDK2, CDK4 and CDK6, were quantitatively determined by real-time PCR using 7300 Real Time PCR System (Applied Biosystems, Singapore), with SYBR Green (Toyobo Co., Osaka, Japan). The relative abundance of the above-mentioned mRNA was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA for quantitative evaluation. The sequences of the primers are given in Table 1. The results were confirmed by at least two independent experiments.

Transfection with siRNA The siRNA duplexes corresponding to GADD45 and 153 were prepared by Invitrogen. The siRNA targeting GADD45 corresponds to the sequence GAGCAGAAGACCCGAAAAGGAUGGAUA, and the siRNA targeting GADD153 corresponds to the sequence GAGAAUA-GAGCGUCUACGAGAAA. A blast search against the human reference mRNA sequences revealed that the sequences are not comparable with any other gene transcripts. Stealth RNAi negative control duplex (Invitrogen) was used as a control. PC-9 cells were treated with the siRNA duplexes in the presence of Lipofectamine RNAi max (Invitrogen), according to manufacturer’s instruction, and the final concentrations of siRNA were 10 nM. Cells were treated with curcumin or vehicle 24 h after transfection, and then cells were subjected to quantitative real-time PCR, Western blotting, and DAPI staining for apoptosis assay. The experiments were repeated at least three times.

Western Blotting Cells were treated with various concentrations of curcumin or vehicle for 24 h. After incubation, cells in ice cold PBS were directly lysed in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by

Table 1. The Sequences of the Primers Used in Quantitative Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Forward (5’–3’)</th>
<th>Sequence Reverse (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGGTATCGTGGAAAGGACTCATGAC</td>
<td>ATGCCAGTGAGCTTCCCGTTCAGC</td>
</tr>
<tr>
<td>GADD45</td>
<td>GCCCTGAGTGGTGGACAGAA</td>
<td>CCCACACTTACATCCTTT</td>
</tr>
<tr>
<td>GADD153</td>
<td>AGAGACCCGAAAGCAGAAAGA</td>
<td>TCTCCCTCATGCGTGTCTTT</td>
</tr>
<tr>
<td>p21</td>
<td>TGAGGACTCTCAGGGTGCAA</td>
<td>GGGCCTTGGAGTGTTGAAATC</td>
</tr>
<tr>
<td>p27</td>
<td>TCAGCAACGGGCAGATTCTCTTACT</td>
<td>GGCGCTCTGTCGCCACAGA</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>CGCCCCCACCCTCAG</td>
<td>CCGCAACACACCTAGACT</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>TGCAACTTCTCCCACACAC</td>
<td>AGACGAGCAGAATTACAA</td>
</tr>
<tr>
<td>Bax</td>
<td>AGAGGATGTGGCGCCCGT</td>
<td>CAACACCACTGTGTTGGAATC</td>
</tr>
<tr>
<td>CDK2</td>
<td>GATGACGAGATGTCAGGAGC</td>
<td>AGCTTGAACACGAGGGTC</td>
</tr>
<tr>
<td>CDK4</td>
<td>ATGTGTGCGGCTGTGGA</td>
<td>CACCACTGGTATGCAGTAGCA</td>
</tr>
<tr>
<td>CDK6</td>
<td>GGCCTATGGGAAAGGT</td>
<td>CGTTCAGGCCACAGAA</td>
</tr>
</tbody>
</table>
SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated overnight at 4°C with specific primary antibodies. After incubation, the membranes were treated with horseradish-peroxidase conjugated secondary antibody, and visualized with commercial chemiluminescent detection kit (Pierce Biotechnology, IL, U.S.A.). The results were confirmed by at least three independent experiments.

**Statistical Analysis** Statistical analyses were conducted with Student’s *t*-test. The results were considered to be significant when *p* value was less than 0.05, *p* < 0.05, **p** < 0.01.

**RESULTS**

**Up-Regulation of GADD45 and 153 by Curcumin**

The treatment of PC-9 cells with curcumin strongly induced expression of both GADD45 and 153 in dose- and time-dependent manner. Specifically, the expression of GADD45 and 153 in PC-9 cells treated with 50 μM curcumin increased 27.4 and 20.6 fold, after 24 h (Fig. 1A). To determine the p53 dependency in the up-regulation of GADD genes, we used human lung cancer cells A549, which have wild type p53. The treatment of A549 cells with curcumin also increased the expression of GADD45 and 153, compared with vehicle, suggesting that the up-regulation of GADD45 and 153 by curcumin was p53-independent.

Based on the evidence that curcumin strongly induced the expression of GADD45 and 153 in PC-9 cells, we next studied whether curcumin could induce their protein production, determined by Western blotting: Consistent with the gene expression, treatment of the cells with 50 μM curcumin clearly increased the protein level of GADD45 and 153 (Fig. 1B).

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**Fig. 1. Up-Regulation of GADD45 and 153 by Curcumin**

Cells were treated with various concentrations of curcumin or vehicle and then (A) gene expression was determined by real-time PCR. Bars represent mean±S.D. of two representative experiments performed in triplicate. **p** < 0.01. Expression level of genes normalized to GAPDH of control cells was calculated as 1. (B) analyzed for protein expression. Actin was used as loading control. Three independent experiments produced similar results.
Inhibition of Cell Growth by Curcumin  The treatment of PC-9 cells with various concentrations of curcumin inhibited cell growth in dose-dependent manner, as shown in Fig. 2. Specifically, the percentage of viable cells 24 h after treatment with 50 μM curcumin was 47.5% of the vehicle control, and treatment with 25 and 50 μM curcumin after 48 h reduced viable cells to 68.3 and 33.5% of control.

Cell Cycle Arrest of PC-9 Cells  We studied the regulatory effects of curcumin on the cell-cycle. Treatment of PC-9 cells with 50 μM curcumin significantly increased the cells of G1 phase from 48.0 to 62.0% (Fig. 3, Table 2). However, curcumin at concentrations of 12.5 and 25 μM caused accumulation of the S phase at 24 h, from 21.5 to 36.0% and 42.7% (Fig. 3, Table 2). We think curcumin induced the G1 and S phase arrests in the cell cycle, resulting in the growth inhibition of PC-9 cells.

Apoptotic Induction of PC-9 Cells by Curcumin  To investigate the mechanisms of the growth inhibition, we de-

Table 2. Induction of G1/S Cell-Cycle Arrest by Curcumin

<table>
<thead>
<tr>
<th>Curcumin (μM)</th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
<th>Sub G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48.0±3.0</td>
<td>21.5±3.9</td>
<td>25.8±1.6</td>
<td>4.7±0.6</td>
</tr>
<tr>
<td>5</td>
<td>37.8±1.3</td>
<td>29.8±1.6</td>
<td>28.2±1.4</td>
<td>4.2±0.3</td>
</tr>
<tr>
<td>12.5</td>
<td>32.8±5.0</td>
<td>36.0±5.3</td>
<td>26.8±0.3</td>
<td>4.3±0.6</td>
</tr>
<tr>
<td>25.0</td>
<td>25.3±5.1</td>
<td>42.7±6.7</td>
<td>22.5±0.9</td>
<td>10.8±1.4</td>
</tr>
<tr>
<td>37.5</td>
<td>41.5±3.0</td>
<td>22.0±2.2</td>
<td>17.7±2.3</td>
<td>17.3±1.2</td>
</tr>
<tr>
<td>50.0</td>
<td>62.0±1.0</td>
<td>4.3±1.1</td>
<td>15.0±1.0</td>
<td>18.3±0.6</td>
</tr>
</tbody>
</table>

PC-9 cells were treated with various concentrations of curcumin for 24h, stained with PI and then analyzed for DNA content by flow cytometry. Values are mean±S.D. of two independent experiments.

Fig. 2. The Effect of Curcumin on the Growth Inhibition of PC-9 Cells

Cells were treated with various concentrations of curcumin. Growth inhibition was measured by trypan blue dye exclusion test after 24 and 48 h. Results are representative of three separate experiments performed in quadruplicate; values are mean±S.D. *p<0.05. **p<0.01.

Fig. 3. Induction of Cell Cycle Arrest by Curcumin

PC-9 cells were treated with various concentrations of curcumin for 24h, stained with PI and then analyzed for DNA content by flow cytometry. Two independent experiments produced similar results.
Fig. 4. Induction of Apoptosis by Curcumin

PC-9 cells were treated with various concentrations of curcumin. After 48 h, (A) cells were treated with DAPI and morphological changes corresponding to apoptosis were measured by fluorescence microscopy. Results are representative of three separate experiments performed in quadruplicate; bars are mean±S.D. ***p<0.01. (B) Cells were treated with Annexin V-FITC/PI double staining, and percent of apoptotic cells was determined by flow cytometry. Two independent experiments produced similar results.
terminated apoptosis of PC-9 cells by morphological changes with DAPI staining. Treatment with various concentrations of curcumin dose-dependently increased the percentages of apoptotic cells with condensed and fragmented nuclei: Curcumin at 5, 12.5, 25, 37.5 and 50 µM concentrations induced 5.8, 12.0, 32.0, 39.7 and 59.8% of apoptotic cells, respectively, after 48 h (Fig. 4A). Furthermore, induction of apoptotic cells was confirmed by FITC conjugated Annexin V and PI double staining. As shown in Fig. 4B, curcumin dose-dependently increased the numbers of Annexin V positive PC-9 cells. These results with curcumin-treated cells clearly demonstrated the expression of GADD genes, growth inhibition, and induction of cell-cycle arrest and apoptosis.

**Alteration of Cell Cycle and Apoptosis Related Gene Expressions by Curcumin**

Cell-cycle is well regulated by cyclins and their cyclin-dependent kinases, and conversely modulated by cyclin dependent kinase inhibitors. To study the mechanisms of these cell-cycle regulatory genes in PC-9 cells treated with curcumin, the expression of *cyclin D1, CDK2, CDK4* and *CDK6* genes was determined by quantitative real-time PCR: After 24 h, mRNA levels of all genes decreased by treatment with curcumin in PC-9 cells, as shown in Fig. 5. Specifically, at 50 µM concentration of curcumin, the mRNA levels of *cyclin D1, CDK2, CDK4* and *CDK6* were down-regulated 0.21, 0.35, 0.43 and 0.58 fold after 24 h, compared with that of vehicle. However, treatment with 50 µM curcumin significantly increased the mRNA levels of the CDK inhibitors *p21* and *p27* to 15.0 and 4.76 fold, after 24 h (Fig. 5). Treatment with curcumin decreased the expression of *Bcl-2* (Fig. 5), but did not change the expression of *Bax* (data not shown).

**Inhibitory Effects on Expression of GADD and 153 by siRNAs**

Our results indicated that curcumin induces the up-regulation of GADD45 and 153 and their protein production, resulting in apoptotic induction. We studied the roles of GADD45 and 153 in the mechanisms of apoptotic induction by employing siRNA duplexes, which selectively inhibit the expression of GADD45 and 153 in PC-9 cells: At 24 h, treatment of PC-9 cells with GADD45 and 153 siRNAs inhibited the expression of GADD45 and 153 induced by 50 µM curcumin (Fig. 6A). The siRNAs also clearly inhibited the protein production of GADD45 and 153 in PC-9 cells induced by curcumin (Fig. 6B).

Next we looked at whether the reduction of GADD45 and 153 proteins would have any effect on the apoptotic induction by curcumin in PC-9 cells. In contrast to the mock experiments, control siRNA induced apoptosis in 25.0 and 34.2% of PC-9 cells treated with 25 and 50 µM curcumin, respectively, at 24 h (Fig. 6C). However, treatment with GADD45 and 153 siRNA showed much lower sensitivity in the apoptotic induction by curcumin: 11.8 and 12.9% at 25 µM, and 15.5 and 14.6% at 50 µM, respectively (Fig. 6C).

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**Fig. 5. Curcumin Altered Cell Cycle and Apoptosis Related Genes in PC-9 Cells**

Cells were treated with various concentrations of curcumin or vehicle, and gene expression was determined by real-time PCR. Bars represent mean±S.D. of two representative experiments performed in triplicate. *p<0.05. **p<0.01. Expression level of genes normalized to *GAPDH* of control cells was calculated as 1.
The results indicate that GADD45 and 153 play significant roles in the induction of apoptosis by curcumin.

DISCUSSION

Our current study revealed that curcumin significantly inhibits the growth of human lung cancer cell PC-9, and induces G₁ and S phase arrests in cell-cycle regulation and apoptosis. The results of G₁ arrest are quite consistent with previous reports showing that curcumin induces anti-proliferative effects through G₁ arrest in various cancer cells, such as human breast and prostate cancer cells. We also found that the cell-cycle arrest by curcumin is associated with down-regulation of various genes, such as cyclin D1 and the cyclin dependent kinase CDK2, CDK4 and CDK6. Cyclin D1 is an essential regulator of cell-cycle in G₁/S phase transitions and its function is disrupted in most cells of human cancer. Increased expression of cyclin D1 was also found in various types of cancer, such as non-small cell lung carcinoma (NSCLC), and the results were further supported by evidence that a deficiency of cyclin D1 protein decreases the development of squamous cell carcinomas. It is well established that the G₁/S transition is regulated by the complex of D1 cyclin and CDK4/6: We think that the treatment with curcumin induces G1 arrest in PC-9 cells by the down-regulation of genes cyclin D1 and CDK4/6, and that it also decreases the expression of gene CDK2, the results of which demonstrate an important role of CDK2 in G₁ checkpoint.

p21, also known as Cip1 or WAF1, is a 21 kDa protein of the family of universal cyclin/CDK inhibitors; it binds simul-
p27/Kip1 is another member of the CDK inhibitor family, which is involved in the transition of G1 to S phase, mediated through the binding of p27 to CDK2 and cyclin E2 complexes, resulting in tumor suppression. Several studies have shown that the lack of functional p27 causes the development of tumors in humans. 32—35) In our study, we demonstrated the increased expression of p27 in PC-9 cells treated with curcumin, suggesting that the up-regulation of p27 is associated with cancer preventive activity of phytochemicals. 21)

We examined the knockdown of GADD45 and 153 gene expression by siRNA in PC-9 cells. However, the significant changes of the gene expression on cell-cycle regulators such as p21, p27, cyclin D1, CDK2, CDK4, and CDK6 were not observed (data not shown). Based on our results, we assumed that the observed G1/S cell-cycle arrest by curcumin in PC-9 cells is related to the alteration of cell-cycle regulators by curcumin.

It is of importance to note that the treatment with curcumin up-regulated the expression of GADD45 and 153 in both p53 mutated PC-9 cells and p53 wild type A549 cells, indicating that p53 protein does not have any effect on expression of GADD45 and 153. The experiments of Scott et al. indicated that GADD gene expression is independent of p53, since GADD45 and 153 gene expressions by deoxycholate are induced in both HCT-116 colonocytes with wild type p53 and p53 mutant HCT-15 colonocytes. 36) Thus, we think that the gene expression by deoxycholate in colonocytes and that in PC-9 and A549 cells by curcumin would be induced similarly independent of p53. Numerous studies have found a correlation between up-regulation of GADD45 and 153 and apoptotic induction. 55—58) In one study, the induction of apoptosis by hyperoxia was reported to be associated with up-regulation of GADD45 and 153 in mice. 40) In our present study, we showed that treatment with siRNAs inhibited the expression of GADD45 and 153 in PC-9 cells and reduced the role of curcumin in the induction of apoptosis, suggesting significant roles of the GADD gene family in the mechanisms of cancer prevention in PC-9 cells by treatment with curcumin. A schematic illustration of mechanism of action for curcumin was presented in Fig. 7.

Turmeric is widely consumed in various parts of the world and is common in the Indian subcontinent as a dietary spice, and curcumin is the most active component of turmeric. In a phase I clinical trial, it was reported that curcumin is not toxic for humans even at a dose of 8 g/day. 41) The average daily intake of turmeric is about 2.0—2.5 g in some countries as a dietary spice, which results in up to 100 mg of curcumin ingestion on a regular basis without any side effects. 42) Taken together, GADD45 and 153 are new targets for curcumin in lung cancer, and we anticipate the use of curcumin in lung cancer prevention and treatment for humans.

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