

## RESEARCH ARTICLE

# Comparison between Effects of Free Curcumin and Curcumin Loaded NIPAAm-MAA Nanoparticles on Telomerase and PinX1 Gene Expression in Lung Cancer Cells

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### Abstract

**Background:** Herbal compounds such as curcumin which decrease telomerase and gene expression have been considered as beneficial tools for lung cancer treatment. In this article, we compared the effects of pure curcumin and curcumin-loaded NIPAAm-MAA nanoparticles on telomerase and PinX1 gene expression in a lung cancer cell line. **Materials and Methods:** A tetrazolium-based assay was used for determination of cytotoxic effects of curcumin on the Calu-6 lung cancer cell line and telomerase and pinX1 gene expression was measured with real-time PCR. **Results:** MTT assay showed that Curcumin-loaded NIPAAm-MAA inhibited the growth of the Calu-6 lung cancer cell line in a time and dose-dependent manner. Our q-PCR results showed that the expression of telomerase gene was effectively reduced as the concentration of curcumin-loaded NIPAAm-MAA increased while expression of the PinX1 gene became elevated. **Conclusions:** The results showed that curcumin-loaded- NIPAAm-MAA exerted cytotoxic effects on the Calu-6 cell line through down-regulation of telomerase and stimulation of pinX1 gene expression. NIPAAm-MAA could be good carrier for such kinds of hydrophobic agent.

**Keywords:** Curcumin - NIPAAm-MAA - PinX1 - telomerase - lung cancer cells

*Asian Pac J Cancer Prev*, 15 (20), 8931-8936

### Introduction

Lung cancer remains the leading cause of cancer-related deaths in both men and women worldwide and in Iran, is one of the five leading tumours. This cancer increasingly common in women (Brown et al., 1996; Bach et al., 1999; Yoder 2006; Molina et al., 2008). The 2 main types of lung carcinoma are small cell lung cancer (SCLC) and non-SCLC (NSCLC); NSCLC accounts for approximately 85% of all cases of lung cancer (Molina et al., 2008). It accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008 (Jemal et al., 2011). In females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. One of the important risk factors for lung cancer is smoking. Smokers have a 20-fold increased risk of developing lung cancer than never-smokers, with 85%-90% of all lung cancers being directly linked to tobacco exposure. The complicated scientific basis for the relationship between smoking and lung cancer continues to be investigated and unraveled. Occupational

exposure to carcinogens accounts for 9%-15% of lung cancer cases (Hosseini et al., 2009). Smoking accounts for 80% of the worldwide lung cancer burden in males and at least 50% of the burden in females. Smoking avoidance could almost completely eliminate the disease. Recent studies demonstrated that expression of human telomerase alone is sufficient for the immortalization of diverse cell types. This enzyme activity is observed in more than 85% in the most cancer cells and is critical for cancer cell growth (Kazemi et al., 2013). Telomerase is a ribonucleoprotein reverse transcriptase which maintains telomeric ends of eukaryotic chromosomes during DNA replication. Telomerase composed of a single-stranded RNA (hTR) which serves as a template for telomeric DNA synthesis and a catalytic protein subunit (hTERT) with reverse transcriptase activity, which adds a template region of the hTR RNA onto chromosomal ends as the telomeric DNA sequences. hTERT is highly expressed in all tissues regardless of telomerase activity, but in cancer cells generally have fivefold-higher expression. Therefore, targeting the telomerase in cancers could

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be promising step in its treatment (Cong et al., 2002). PinX1 has a unique property to directly bind to TERT and TERC and inhibit telomerase activity at telomere. Furthermore, PinX1 inhibition in cancer cells activates telomerase and elongates telomeres whereas PinX1 over expression has the opposite effects (Hosseini et al., 2009). PinX1 is a nucleolar protein evolutionarily conserved from the yeasts to the human being and PinX1 gene localizes to human chromosome 8p23, which is one of the regions that experiences the most frequent loss of heterozygosity, (LOH) in many common human carcinomas. In recent years, the focus of cancer control has been on the search for anticancer agents, which are safer and have higher acceptability for patients. In this regards, various natural agents such as turmeric and polyphenolic, which are generally a part of human diet or traditional herbal medications, have been taken attention (Nasiri et al., 2013). One of the most natural compounds is Curcumin (CUR) (Kazemi-Lomedasht et al., 2013). Curcumin is a natural compound primarily used for food colouring and obtained from the herb of *Curcuma longa*, has been recognized as a chemopreventive agent because of its antitumor and antiproliferative effects. It regulates an array of cellular processes such as inhibition of lipid peroxidation, nitric oxide synthetase activity, epidermal growth factor (EGF) receptor kinase C activity, NF- $\kappa$ B activity, protein kinase C activity and production of reactive oxygen species (Motterlini et al., 2000; Aggarwal et al., 2005; Ramachandran et al., 2005; Li et al., 2013). Pre-clinical and clinical studies showed that curcumin possessed several disadvantages such as instability, poor bioavailability and fast metabolism (Liang et al., 2009). The last decade has witnessed enormous advances in the development and application of nanotechnology in cancer detection and therapy culminating in the development of the nascent field of "cancer nanomedicine" (Azarmi et al., 2008; Li et al., 2014). Nanoparticle-based therapeutic systems have gained immense popularity due to their ability to overcome barriers, effectively deliver hydrophobic therapies, and target disease sites. Nanoparticle delivery to the lungs is an attractive concept because it can cause retention of the particles in the lungs accompanied with a prolonged drug release if large porous nanoparticle matrices are used (Azarmi et al., 2008). Drug loaded polymeric nanoparticles offer several favorable biological properties, such as biodegradability, no toxicity, biocompatibility and mucoadhesiveness. Hydrogels are one of the upcoming classes of polymer-based controlled release drug delivery systems. Besides illustrating swelling-controlled drug release, hydrogels also exhibit stimuli-responsive changes in their structural network and therefore, the drug release (Gupta et al., 2002) Temperature-sensitive hydrogels are probably the most commonly studied class of environmentally sensitive polymer systems in drug delivery research. The common characteristic of temperature-sensitive polymers is the presence of hydrophobic groups, such as methyl and propyl groups (Yadav et al., 2014). As the representative one, poly (N-isopropylacrylamide) (PNIPAAm) is a well known temperature sensitive polymer, which exhibits phase separation at a lower critical solution temperature

(LCST) of  $\sim 32^{\circ}\text{C}$  in aqueous solution. Below this temperature, PNIPAAm is hydrophilic and exists in chain with a coil conformation; while above the LCST, it undergoes a sharp coil-to-globule transition to form inter- and intrachain association, resulting in hydrophobic aggregation and depositing from the aqueous solution. Up to now, because of this reversible phase transition, PNIPAAm has been widely used to prepare temperature sensitive hydrogels (Wang et al., 2008; Davaran et al., 2014). From the viewpoint of hydrogel applications, it would be favorable if the hydrogel could respond to two types of stimuli simultaneously, either mutually or independently, with particular emphasis on the pH and temperature stimuli. The pioneer work was done by Chen and co-workers, who grafted PNIPAAm to poly (acrylic acid) (PAAc) chain to obtain copolymers exhibiting temperature-induced phase separation over a wide range of pH values. Subsequently, to achieve pH and temperature sensitive hydrogels, copolymerizing the temperature sensitive NIPAAm with other co-monomers such as methacrylic acid (MAA) has drawn much attention (Wang et al., 2008). The pH-responsive polymeric nanoparticles such as NIPAAm-MAA could release the drug in a slightly acidic environment, which occurs in, inflammatory tissues, solid tumors and intracellular endosomal compartments (Tian et al., 2008). In this study, we comprise the effect of pure Curcumin and Curcumin-loaded NIPAAm-MAA nanoparticle on Telomerase and PinX1 genes expression in lung cancer cell line.

## Materials and Methods

Materials for supply medium culture inclusive RPMI 1640, Fetal Bovine Serum (FBS), and Antibiotics along Tripsin-EDTA and Trizol-reagent were Purchased from Invitrogen (Germany). Syber Green Real Time PCR Master Mix kit was Purchased from Roche (Germany). (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Sigma-Aldrich. RNXplus kit and c-DNA kit Synthesis, were purchased from. Curcumin were purchased from Sigma-Aldrich, Stock solutions of curcumin (5 mM) were prepared freshly on the day of the experiment by dissolving the compound in ethanol. Calu-6 lung cancer cell line was prepared from Pasteur Institute cell bank of Iran.

Cell culture and cell line: Calu-6 lung cancer cell line was prepared from cell bank of Iran and was grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate (2 mg/ml), penicillin G (0.05 mg/ml), streptomycin (0.08 mg/ml) in a humidified air containing 5%  $\text{CO}_2$ .

### Preparation of NIPAAm-MAA copolymers particle

Poly (NIPAAm-MAA) copolymers were synthesized by free radical polymerization of monomers in 1,4-dioxane under  $\text{N}_2$  atmosphere. Water soluble monomers, NIPAAm, and MAA, were used in 8:5:0.3 molar ratios and the polymer were cross-linked with DMTEMA. Also 0.17 gr benzoyl peroxide (BPO) as initiator was added to solution. The polymerization was carried out at  $70^{\circ}\text{C}$  for 24 hour under  $\text{N}_2$  atmosphere. Polymer was obtained

by precipitation in an excess amount of cold n-Hexan.

#### Scanning electron microscopy (SEM)

Shape, size, and aggregation of NIPAAm-MAA were observed by scanning electron microscopy (SEM). The nanographs of NIPAAm nanoparticles and NIPAAm-Curcumin are shown in Figure 1. As it is demonstrated the size of the particles is about 40-50 nm and dispersion of the particles was greatly improved.

#### Preparation of curcumin-loaded NIPAAm-MAA

Nanoprecipitation technique was used to prepare the curcumin-loaded NIPAAm-MAA. 120 mg lyophilized powder of NIPAAm-MAA polymeric was dispersed in 10 ml of water and was stirred well. Curcumin was dissolved in absolute ethanol (2-3 mg/ml). Curcumin got directly loaded into the NIPAAm-MAA. The drug loaded polymeric were then lyophilized 24 h get dry powder for subsequent use.

#### MTT assay

The MTT assay is an important method for evaluating the cytotoxicity of biomaterials *in vitro*. The cytotoxic activity of free curcumin and encapsulated were assessed by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide and in the triplicate model. Briefly, Cells (1. 10<sup>3</sup> cells per well) were seeded in 96-well plates. After 24 h incubation in 37°C with humidified atmosphere containing 5% CO<sub>2</sub>, the cells were treated with serial of different concentrations of free (dissolved in ethanol) (10,20,30,40,50,60,70 μM) and equivalent doses NIPAAm-encapsulated Curcumin for 24,48,72. In addition, cells with 200 μl culture medium containing FBS was served as control. After incubation, medium of all wells were exchanged with fresh medium and cells were leaved for 24 h in incubator. Then, medium of all wells were removed and 50 μl of 2 mg/ml MTT dissolved in PBS was added to each well and incubated for 4 hours. After removing content of the wells, 200 μl pure Dimethylsulfoxide (DMSO) was added to each well to dissolve blue formazan precipitate. Then, 25 μl Sorensen's glycine buffer was added. Finally, the plates were shaken and absorbance of each wells was read in 570 nm using ELISA plate Reader with reference wavelength of 630 nm. The cell viability was expressed as a percentage of the control by the following equation: Viability % = Nt/N0 where Nt is the absorbance of the cells treated with free curcumin or curcumin-loaded NPs and N0 is the absorbance of the untreated cells.

#### Cell treatment

After determination of IC<sub>50</sub> by MTT Assay, 1×10<sup>6</sup> cells were treated with serial concentrations of curcumin pure and NIPAAm-curcumin. For control cells, the same volume of 10% DMSO without drugs was added to flask of control cells. Then, culture flasks were incubated in 37°C containing 5% CO<sub>2</sub> with humidified atmosphere incubator for 24, 48 and 72h exposure duration.

#### *In vitro*-release studies

To study drug release, four different sets of

experiments were performed. These included two temperatures (40°C and 37°C) and two pH levels (5.8 and 7.4). In each drug-release experiment, 3.0 mg of the drug carrier bonded with smart polymer was sealed in a 30 mL of Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> buffer solution at a pH of 5.8 or 7.4. The test tube with a closer was placed in a water bath maintained at 40°C up to the lower critical solution temperature or 37°C (higher than the lower critical solution temperature). The release medium (about 3 mL) was withdrawn at predetermined time intervals (1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 24, 36, 48, 60, 80, 100, 130, 160, 190, and 220 hours). Thereafter, the samples were analyzed using an ultraviolet-visible spectrometer (Shimadzu) to determine the amount of Curcumin released.

#### RNA Extraction and cDNA synthesis

For extraction of RNA Trizol isolation reagent (Invitrogen, USA) was used according to the manufacturer's protocol for cell lines. After extraction of RNA, Sample RNA content was quantified by measuring absorbance at 260 nm. Then, the firmness of extracted RNA was defined by electrophoresis in 0.5 μg/ml ethidium bromide contained agarose gels. Complementary DNA (cDNA) was synthesized using random primers (N6) and hexamer primers with purchased reverse transcriptase kit from fermentas.

#### Real-time PCR (qRT-PCR) assay

Quantitative Real-time PCR technique was applied for appointment of Scale of hTERT and Pinx1 genes expression. The forward (F) and reverse (R) primer sequences of pinX1 and hTERT that used in real-time PCR were (5'CACTCCAGAGGAGAACGAAACC3', 5'CACCGGCTTGCAAAGTACT3' respectively) for pinX1 (166 bp) and (5'GTCTGGAGCAAGTTGCAAA G3'5'TGACCTCTGCTTCCGACA3' respectively) for hTERT (198 bp). These primers were blasted by primer-blast site on NCBI website. In a 25 μl reaction mixture that contained: 5 pmole of the forward and reverse PCR primers of hTERT and pinX1, 2X PCR Master Mix Syber Green I, and 2 μl of the cDNA were a bp amplicon of hTERT and a bp amplicon of pinX1 were generated. The Beta-Actin mRNA was calculated as the internal standard control gene by specific primers. The quality of real-time PCR reactions was controlled by running standard samples as duplicated. Negative controls were prepared each time, consisting of 2 μl ddH<sub>2</sub>O instead of the cDNA template. The sample tubes were placed into the (Rotor-Gene 6000, Corbet) with the following settings as manufacture protocol (Table 1).

#### Statistical analysis

SPSS 16 has been used for statistical analysis and p value <0.05 was regarded statistically significant.

## Results and Discussion

#### Determination of drug content of the nanoparticles

To determine the efficacy of drug loading, 100 mg dried powder of nanoparticles was dissolved in 100 ml phosphate buffer (pH = 7.2). After complete dissolution

**Table 1. The PCR Program for hTERT, PinX1 and Beta Actin**

Step	Temperature	Time	Number of cycles
Holding	95	5min	1
Denaturation	95	15sec	-
Annealing	60	15sec	40
Extension	72	30sec	-
Melting	70-95	-	1

of curcumin- loaded polymer, the amount of drug in the solution was quantified using UV spectrophotometer at  $\lambda_{max} = 420$  nm. Drug loading capacity (DL %) was calculated as drug analyzed in the nanoparticles versus the total amount of the drug and the polymer, according to the following equation:

$$DL (\%) = \frac{\text{Amount of drug loaded in nanoparticles} \times 100}{\text{Amount of nanoparticles}}$$

In this study, Drug loading capacity (DL %) was acquired 89. 6%.

**Drug release**

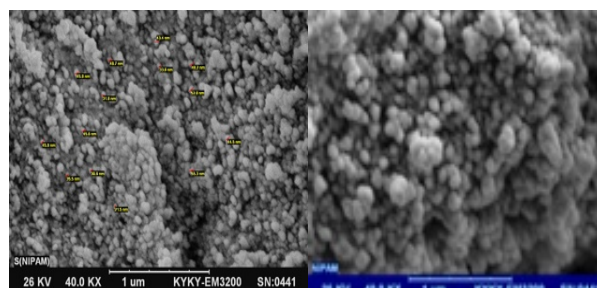
After 220 hours in phosphate buffer solution (pH 7. 4, 5. 8) at 37°C and 40°C, the release behavior of the nanoparticles was studied. Our results demonstrated the percentage of cumulative release of Curcumin at 40°C and PH=5. 8 was significantly higher than at 37°C and PH=7. 4 (Figure 2).

**Spectral characterization of NIPAAm–MAA nanoparticles**

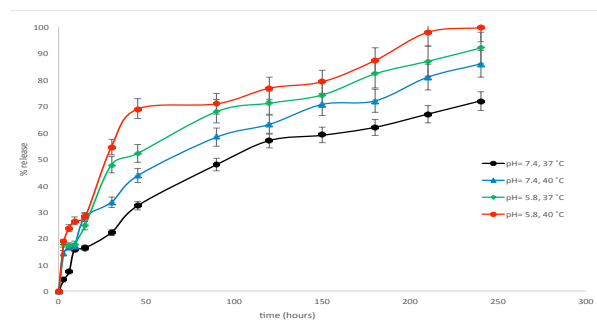
The chemical structure of the cross-linked Poly (NIPAAm-MAA) was characterized by FT-IR spectroscopies. Figure 3 shows the FT-IR spectrum of the copolymer and copolymer with Curcumin. As exhibited in the FT-IR spectrum of the copolymer,absorbance of amide carbonyl group in NIPAAm appears at 1660  $cm^{-1}$ , bending frequency of amide N-H appears at 1520  $cm^{-1}$ . The most important bands of MAA are at: 3350  $cm^{-1}$  [ $\nu$  (OH)], 2950  $cm^{-1}$  [ $\nu$  (CH3) as,  $\nu$  (CH2) as], and 1080  $cm^{-1}$  [ $\nu$  (O-C), alcohol], 1040  $cm^{-1}$  [ $\nu$  (O-C) ester], and 720 [ $\nu$  bending (O=C-O)]. The FTIR result indicated successful polymerization of NIPAAm and MAA. The FTIR spectra of PNIPAAm-MAA-Curcumin using shows following characteristic signals at: (a)1627  $cm^{-1}$  which is a characteristic peak for C=O (enolic), (b) 1520  $cm^{-1}$  shows the presence of C. C group, (c) 1250  $cm^{-1}$  shows the C. O stretching, and (d) 3547  $cm^{-1}$  shows the presence of OH group present in the molecule, respectively, corresponding to the presence of curcumin loaded PNIPAAm-MAA nanoparticles.

**Results of MTT assay**

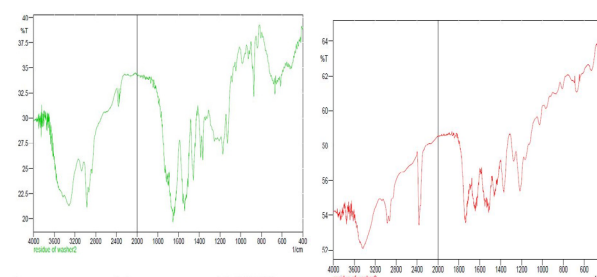
Different concentrations of pure curcumin and NIPAAm-Curcumin (10~70 $\mu$ mol/L) at different time intervals 72, 48 and 24 h had cytotoxicity effects on Calu-6 cells in a dose dependent manner, as determined by the MTT assay. Data analysis of the cytotoxicity assay showed that IC50s of effect of Free curcumin and NIPAAm-Curcumin on Calu-6 lung cancer cell line are



**Figure 1. SEM Pictures of Particles: A (NIPAAm-MAA) and B(NIPAAm-Curcumin)**



**Figure 2. Release of Curcumin at Different Temperature and PH**

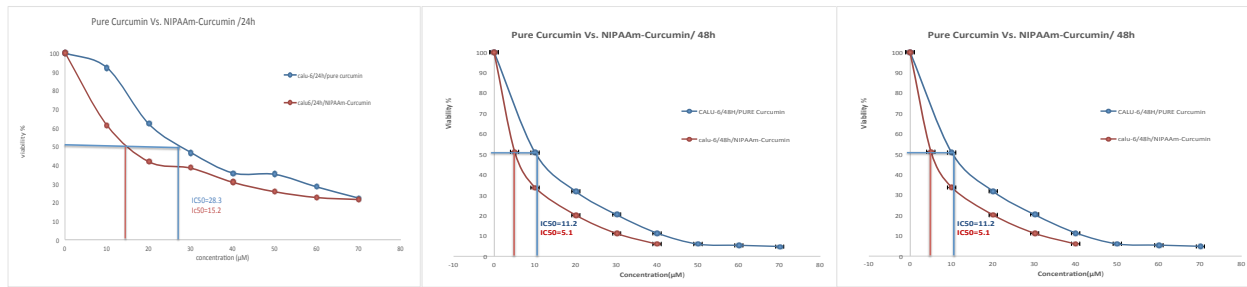


**Figure 3. FT-IR Spectra the Structure of Expected A) NIPAAm-MAA and B) NIPAAm -Curcumin**

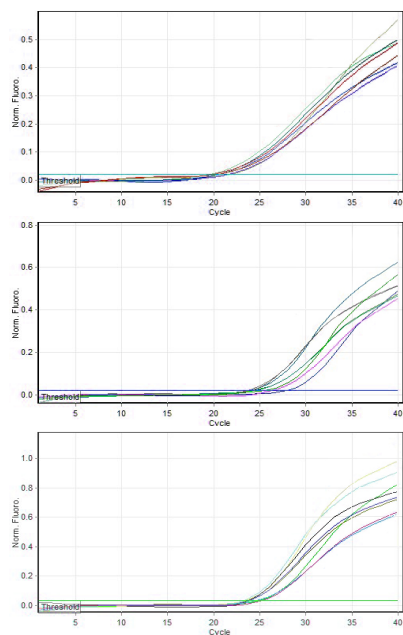
27, 16 $\mu$ M for 24 h ( Figure 4A),12,7 $\mu$ M for 48 h (Figure 5B) and 7, 6 $\mu$ M for 72 h MTT assays respectively (Figure 5C). Cells treated with concentrations more than 60  $\mu$ M of Free curcumin and NIPAAm-Curcumin for 48 and 72h were died completely. In the current work, the MTT assay showed that Curcumin -loaded NIPAAm have time-dependent and dose-dependent cytotoxicity in Calu-6 lung cancer cell line. Moreover IC50 show that effect of NIPAAm-Curcumin on cells is more than free Curcumin in defferent time.

**Results for quantitative real-time PCR**

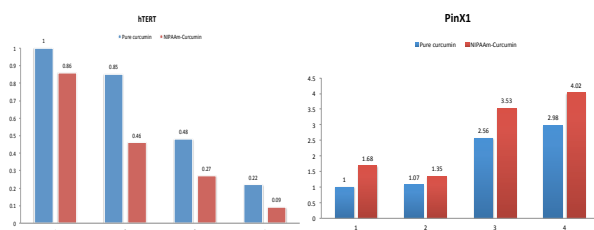
hTERT and Pinx1 mRNA levels were measured via real-time PCR. The level of hTERT mRNA was normalized to mRNA level of the uniformly expressed housekeeping gene, beta actin, within each sample and each sample was repeated two times (Figure 5). Calculation of  $2^{-\Delta\Delta CT}$  values demonstrated With increasing amount of  $2^{-\Delta\Delta CT}$ , the expression of mRNA levels decreases. Figure 6 demonstrate that with increasing concentration NIPAAm-Curcumin a decreasing trend was appeared in mRNA levels of hTERT and with increasing concentration NIPAAm-Curcumin a increasing trend was appeared in mRNA levels of pinX1.



**Figure 4. Cytotoxic Effects.** A) pure Curcumin vs NIPAAm-Curcumin IN 24h B) pure Curcumin vs NIPAAm-Curcumin IN 48h C) pure Curcumin vs NIPAAm-Curcumin IN 72h



**Figure 5. Amplification Plot.** A) Beta actin B) hTERT C) PinX1



**Figure 6. Level of hTERT and PinX1 mRNA expression in cells treated with Pure Curcumin and NIPAAm-Curcumin**

In conclusion: in this study, we used Curcumin-loaded NIPAAm-MAA and pure Curcumin for determination of their effects on scale of hTERT and pinX1 expression genes in Calu-6 lung cancer cell line. Curcumin, a polyphenol compound is derived from the herb of *Curcuma longa* (known as turmeric) that has inhibitory effect on hTERT and pinX1 genes expression in lung cancer. Despite therapeutic effects of curcumin, its use is limited due to: poor bioavailability, slow dissolution rate, low water solubility, photodegradation in organic solvents, and variability in the gastrointestinal tract. Nanotechnology provides an alternative strategy to overcome these problems by encapsulating or attaching drugs to nonmaterial such as polymers and solid-core

nanoparticles. Hydrogels are one of the upcoming classes of polymer-based controlled release drug delivery systems that show stimuli-responsive changes in their structural network. Poly (N-isopropylacrylamide) (PNIPAAm) is the most popular thermosensitive polymer and could release the drug in a slightly acidic environment, which occurs in, inflammatory tissues, solid tumors and intracellular endosomal compartment. In this study, we evaluated Curcumin-loaded NIPAAm-MAA and pure Curcumin on hTERT and pinX1 genes expression in Calu-6 lung cancer cell line with quantitative Real-time PCR. In this study, the comparison between the effect of pure curcumin and curcumin-loaded NIPAAm on Telomerase and Pinx1 genes expression in Calu-6 lung cancer cell line, Theour results demonstrated Cells treated with NIPAAm-Curcumin and cells treated with pure Curcumin had difference in the levels of telomerase and Pinx1 genes expression also the effect toxicity NIPAAm-Curcumin on cells is higher than pure Curcumin with the same values and under the same conditions. This cytotoxic effect on the cells was increased with increasing concentration of NIPAAm-MAA-curcumin complex and was time and dose-dependent too. It should be noted that the effect NIPAAm-curcumin complex in Calu-6 cell line has never been done so far, and inhibitory effect of NIPAAm-curcumin complex on hTERT and Pinx1 genes expression has never been done so far. So there isn't anything for comparison. Also, the *in vitro* effect of NIPAAm-curcumin complex on Calu-6 cell line requires further investigation.

## Acknowledgements

The authors thank Department of Medical Biotechnology, Faculty of Advanced Medical Sciences of Tabriz University of Medical Sciences for all supports provided. also thak Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.

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