

## RESEARCH COMMUNICATION

# Overexpression of Triosephosphate Isomerase Inhibits Proliferation of Chicken Embryonal Fibroblast Cells

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## Abstract

Triosephosphate isomerase (TPI), a glycolytic enzyme, functions in catalyzing the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate and is generally upregulated in tumours. However, there are data suggesting loss in lymphomas. To determine its effects in chicken embryonal fibroblasts (CEF) a plasmid was constructed to allow transfection. Upon culture in a suitable medium, cells transfected with the TPI demonstrated upregulation and were significantly more susceptible to apoptosis compared to controls with decreased proliferation. These finding therefore render a novel mechanism by which CEF can be triggered to undergo death by upregulation of TPI.

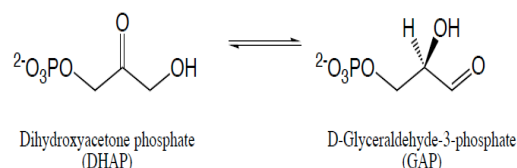
**Keywords:** TPI - overexpression - CEF cells - proliferation

*Asian Pacific J Cancer Prev*, 12, 3479-3482

## Introduction

Triosephosphate isomerase (TIM) is one of the best studied glycolytic enzymes, present in virtually all organisms. It catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) with D-glyceraldehyde-3-phosphate (GAP; Figure 1). The structure of TIM was solved by Petsko in the mid-1970's, at a time when relatively few enzyme structures were known. Triosephosphate isomerase is necessary for efficient energy production, is important in several metabolic pathways, and is present in the cytoplasm (Montgomerie et al., 1997). An inherited deficiency occurs in humans and causes a rise in dihydroxyacetone phosphate. Clinical signs are hemolytic anemia, early death, and neurodegeneration (Olah et al., 2005).

The results of some researchers, finding an absence of triosephosphate isomerase in most lymphoma cases, are in contrast with previous findings of other investigators. Two-dimensional electrophoretic analysis of lung adenocarcinoma revealed an increase of triosephosphate isomerase in 60% of adenocarcinomas compared with uninvolved lung (Chen et al., 2002). Similarly, increased expression of triosephosphate isomerase was found in lung squamous cell carcinomas (Li et al., 2002). Triosephosphate isomerase has also been found to be increased in urinary cancers (Unwin et al., 2003). Possibly, the down-regulation of triosephosphate isomerase in the lymphoma samples represents an underlying cell type specificity, but overall, it seems that misregulation of triosephosphate isomerase is a common feature of cancer.



**Figure 1. The Reaction Catalyzed by Triose Phosphate Isomerase.** Note that the reaction can proceed in either direction

In the present study, we construct pEGFP-N1-TPI, and transfect to CEF cells, to assess effects of overexpression on proliferation.

## Materials and Methods

### Cell lines and cell culture conditions

The primary CEF monolayer was prepared from 10-day-old embryonated specific pathogen-free chicken eggs, and cultured in Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were cultured in an incubator at 5% CO<sub>2</sub> in air at 37°C.

### Construction of an TPI expression vector

Plasmid pEGFP-N1, purchased from Shanghai Cancer Institute, China, was used to construct the TPI expression vector. TPI cDNA PCR product and pEGFP-N1 were digested with PstI and BamHI. The digested PCR product was electrophoresed through and isolated from an agarose gel. After purification, it was ligated into the cut vector

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to form pEGFP-N1. This plasmid was transformed into bacterial competent cells.

#### Establishment of stable transformants overexpressing TPI

The TPI expression vector (pEGFP-N1-TPI) or the empty pEGFP-N1 vector was transfected into CEF and HCT8 cells using FuGENE HD (Promega) according to the manufacturer's protocol. Briefly, each plate was transfected with a mixture containing 5µg of plasmid DNA and 8 ml of FuGENE HD.

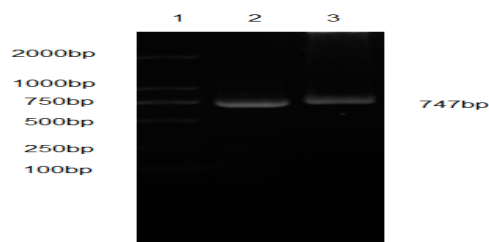
Transfectants were selected by treatment with G418 at 48 h. The concentration of G418 used for selection was gradually decreased as follows: 400 mg/ml for 4 days; 300 mg/ml for 4 days; and 200 mg/ml for maintenance of the cultures. At day 20 after transfection, 11 G418-resistant transformants were established from pEGFP-N1 transfectants, and 15 transformants were established from TPI expression vector transfectants. Stable transformants overexpressing TPI were also selected.

#### RT-PCR analysis

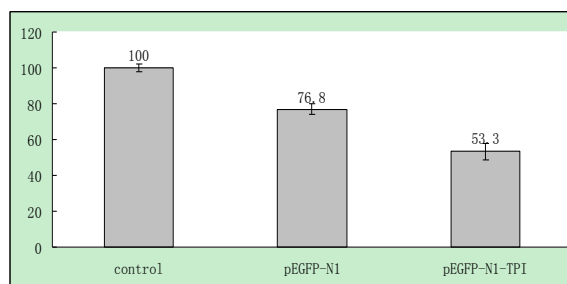
Total cellular RNA was extracted from cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by measuring UV absorption. RNA was reverse transcribed to single-strand cDNA using a Revertaid First Strand cDNA Synthesis Kit (Fermentas International Inc, Canada). The reaction contained 2µg RNA, 1 µl random primer and DEPC-treated water added to the sample to a volume of 12 µl, incubated at 70°C for 10 min and put on ice. Afterwards 4 µl of reaction buffer, 2 µl of dNTPs and 1 ml of RNase inhibitor were added. Samples were incubated at 25°C for 5 min, and added 1 ml of M-MuLV reverse transcriptase was introduced. After incubation at 25°C for 10 min, the samples were finally heated to 48°C for 60 min. The reaction was stopped by heating at 70°C for 10 min, and the samples chilled on ice. The amplification for TPI was done under the following conditions: 25µl reaction mixture contained 2.5µl of PCR buffer, 2µl of dNTP, 2.5 U of TaKaRa LA Taq polymerase (TaKaRa, Japan), 1 µl of each primer (P1: 5'-CTGCAGatggctcccaggaagtcttctgt-3', and P2: 5'-GGATCCggatgttttgcattgataatcca-3'), and 100 ng of cDNA at 94 °C for 5 min; then 94 °C for 45 s, 56°C for 30 s, 72°C for 1 min for 35 cycles, and finally at 72 °C for 10 min for balance. After PCR reaction, 2 µl of the amplified product was electrophoresed in 2% agarose gelatin gels buffered with 1×TBE.

#### Cell proliferation and survival assay

The percentage of growth inhibition was evaluated using a MTT (Sigma) assay for the measurement of viable cells. All the cell lines were seeded onto 96-well plates at a density of 104 cells in a final volume of 100µl, respectively. The TPI expression vector (pEGFP-N1-TPI) or the empty pEGFP-N1 vector was transfected into CEF and HCT8 cells using FuGENE HD (Promega) according to the manufacturer's protocol. 48h later, 20µl of MTT at a concentration of 5mg/ml was added to each of the well, and the cells were incubated for an additional 4h at 37 °C. The supernatants were aspirated and 150µl of DMSO were added to each of the wells, shaken for 10



**Figure 2. RT-PCR of TPI in CEF Cells.** CEF cells were cultured as described in DMEM. Total RNA was isolated and RT-PCR performed with specific primers for TPI. PCR products in CEF/TPI and TPI/N1 cells were separated on 2% agarose gels and stained with ethidium bromide. An obvious TPI band of 747 bp was amplified in CEF/TPI and only a faint band in CEF/N1 cell lines. Lane 1: DL2000marker; Lane 2: CEF/TPI cells; and Lane 3: CEF/N1 cells



**Figure 3. CEF Cell Survival was Assessed by MTT assay.** The results represent the means of at least 3 independent experiments. The mean cell proliferation rate of CEF/TPI cells at transfection after MTT treatment was significantly lower compared with that of CEF/N1 or CEF,  $P < 0.01$

min in order to dissolve any remaining precipitate. The spectrophotometric absorbance was determined using an ELX800 reader (Bio-Tek instruments, Inc., Winooski, VT) at 490 nm wavelength. The mean absorbance of the control wells represented 100% cell survival, and the mean absorbance of treated cells was related to control values to determine sensitivity. The MTT detection assay was repeated in triplicate.

Relative activity (%) =  $[1 - (\text{test-background}) / (\text{control-background})] * 100\%$

## Results

#### Plasmid construction and transfection

To examine the effect of TPI on CEF cell progression in vitro, the plasmid pEGFP-N1 was used to construct the TPI expression vector, pEGFP-N1-TPI. The CEF cell line was engineered to transiently express increased levels of TPI protein, referred to as CEF/N1/TPI. A control cell line was transfected with the empty vector and referred to as CEF/N1. Protein and mRNA levels of TPI in CEF cells were validated by Western blotting with antibody against TPI and reverse transcription-polymerase chain reaction (RT-PCR) with primers specific to TPI, respectively. An obvious 750bp band was observed following gel electrophoresis of RT-PCR products amplified from CEF/TPI cellular RNA, whereas only a faint 750bp band was observed following RT-PCR of CEF/N1 cellular RNA (Figure 2). In addition, markedly higher levels of TPI protein were expressed in CEF/TPI cells. In contrast, less TPI expression was observed in CEF or CEF/N1

cells (Figure 2). The data suggest that the pEGFP-N1-TPI recombinant vector was successfully constructed and TPI was stably overexpressed in CEF/TPI cells in vitro.

#### Overexpression of TPI inhibits proliferation in CEF cells

CEF/TPI cells exhibited significantly reduced cell survival ( $P < 0.01$ ), as assessed by the MTT assay (Figure 3) and CEF/N1 and CEF cells had higher mean proliferation rates, indicative of a suppressive effect of TPI on CEF cell growth and survival.

## Discussion

Most cancer cells exhibit increased glycolysis and use this metabolic pathway for generation of ATP as a main source of their energy supply. This phenomenon is known as the Warburg effect and is considered one of the most fundamental metabolic alterations during malignant transformation. The steady state level of TPI mRNA changes 5- to 20-fold, depending upon the cell type, during the transversal of cells from a proliferative to a nonproliferative state and vice versa (Ationu et al., 1997).

TPI has been selected out by several proteomic studies including metastasis, MDR research and researches of diagnosis and prognosis (Zhang et al., 2005; Svasti et al., 2005; Liu et al., 2006; van den Bemd et al., 2006). However, its functional role has not been fully explained. In Xin's study, they validated the functional role of TPI in drug resistance cell line, and found that its downregulated expression in the drug-resistant SGC7901/VCR cells contributes considerably to its drug resistance phenotype. Enhanced TPI expression can partially reverse MDR phenotype of drug-resistant gastric cells and sensitize tumor cells to chemotherapy, leading to significantly increased ADR accumulation and retention. Previous studies showed that the cellular effects of olomoucine-derived cyclin-dependent kinase inhibitor (CDKI) are not dependent on their ability to inhibit CDKs, but through downregulating TPI, diminish the function of cancer cells in CEM T-lymphoblast leukemia (Kovarova et al., 2000). Thus, it can be delineated that upregulation of TPI could possibly retrieve tumor cells from a resting or dormant fashion back to the cell cycle, and thus sensitize tumor cells to chemodrugs. Further research is currently being carried out to verify this hypothesis.

Some researchers demonstrated that TPI is a direct target molecule of Cdk2 and its catalytic activity was down-regulated by Cdk2-mediated phosphorylation during etoposide-induced apoptosis. Notably, TPI enzyme activity for the reaction from GAP to DHAP was markedly down-regulated when TPI was phosphorylated by Cyclin A/Cdk2 and this down-regulation of TPI activity was prevented by olomoucine, a Cdk2 inhibitor in the phosphorylation reaction of TPI in vitro.

There are several reports demonstrating that TPI is phosphorylated in cultured HeLa cells (Imami et al., 2008), HeLa cells arrested in the mitotic phase of the cell cycle (Dephoure et al., 2008), and also in human pituitary gland (Beranova-Giorgianni et al., 2006). In these studies, it was commonly suggested that serine 21 residue of TPI becomes phosphorylated, but none of them describes

changes in TPI activity.

Cancer cells are, in general, subjected to a partial hypoxic environment, and exhibit increased glycolysis with decreased mitochondrial respiration (Warburg effect), and hence generate the majority of energy required for their growth through glycolysis (Warburg, 1956; Pedersen, 1978; Kondoh, 2008). Therefore, specific inhibition of glycolysis is considered to be a novel therapeutic strategy for treating cancer (Pelicano et al., 2006; Funasaka et al., 2007; Kueck et al., 2007; Clem et al., 2008). TPI catalyzes reversible interconversion of DHAP and GAP in a glycolytic pathway. DHAP that can be utilized as a precursor for gluconeogenesis has to be converted to GAP to enter a glycolytic pathway for the ATP generation under anaerobic conditions. Reduced catalytic activity of TPI through Cdk2-mediated phosphorylation will hence disturb glycolytic pathways, and limit ATP supply in etoposide-treated HeLa cells, leading to apoptosis.

According to recent reports, TPI is nitrosylated in early Alzheimer's disease (Reed et al., 2009) and as a result of nitrosative damage induced by amyloid beta-peptide (Guix et al., 2009). Moreover, nitrotyrosine formation in TPI decreases its isomerase activity and produces methylglyoxalin, a highly neurotoxic chemical. These observations lend support to the notion that post-translational modification of TPI could decrease its activity, which could contribute to damage-induced cell death.

## Acknowledgements

This study was partially funded by the National Natural Science Foundation (30972192), Jilin University, cutting-edge science and interdisciplinary innovation projects (201103223), Zoonosis, Ministry of Education Key Laboratory Fund.

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