Expression of Recombinant Human MT1G Gene with C Terminal of His-tag in EC9706 Cells

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Abstract: Objective. To construct a recombinant eukaryotic expression plasmid of MT1G and express it in human esophageal cancer cell line EC9706. **Methods.** The target sequence was amplified by PCR from pACT2-MT1G plasmid containing human *MT1G* cDNA and cloned into eukaryotic expression vector pcDNA3.1/Myc-His(-) with C terminal of myc epitope and 6×His-tag. After restriction endonuclease digestion and DNA sequencing confirmation, the recombinant plasmid was tranfected into EC9706 cell by lipofectamine 2000. The positive monoclone was screened by G418. RT-PCR and Western blotting were used to detect the expression of mRNA and fusion protein of *MT1G* gene respectively. **Results.** The eukaryotic expression vector pcDNA3.1/Myc-His(-)-MT1G was successfully constructed and MT1G fused protein with His-tag was expressed in tranfected EC9706 cell. **Conclusion.** The human MT1G recombinant plasmid and the EC9706 cell strain stably expressing MT1G fused protein with His-tag were obtained, which provide the basis for further study on biology functions of MT1G. [Life Science Journal. 2006;3(4):49-53] (ISSN: 1097-8135).

Keywords: metallothionein 1G; esophageal cancer; eukaryotic expression

Abbreviations: MTs: Metallothioneins

1 Introduction

Metallothioneins (MTs) are a cystein-rich, low molecular weight proteins, which can bind to heavy metals such as zinc and copper and is involved in their intracellular homeostasis[1]. In humans, MTs are encoded by a family of genes consisting of 10 functional MTs isoforms and the encoded proteins are conventionally subdivided into four groups viz, MT-1, MT-2, MT-3 and MT-4 proteins[2-5]. While a single MT-2A gene encodes MT-2 protein, MT-1 protein comprises many subtypes encoded by a set of MT-1 genes (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H and MT-1X) accounting for the microheterogeneity of the MT-1 protein[6]. Although MTs have been linked with tumorigenesis and progression, there is not much information available in the literature on the functional roles of the different MT isoforms. To investigate the biological roles of MT1G in human esophageal cancer cell line, we constructed the recombinant eukaryotic expression vector pcDNA3.1/Myc-His (-)-MT1G and expressed it in EC9706 cells.

2 Materials and Methods

2.1 Materials

Esophageal cancer cell EC9706, eukaryotic expression vector pcDNA3.1/Myc-His(-), *E. coli.* strain DH5a and strain DH5a containing pACT2-MT1G were from department of Etiology and Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences. Taq DNA polymerase, T4 DNA ligase, *Xba*I and *Bam*HI restriction enzyme, 100 bp Ladder marker, RT-PCR kit were Takara. Lipofectamine™ 2000, TRIZOL reagents were Invitrogen. Rabbit polyclonal His antibody, peroxidase-conjugated secondary antibody were purchased from Beijing Zhongshan Biotechnology Company.

2.2 Methods

2.2.1 Design of primers and amplication of target gene

According to MT1G gene sequences published in GenBank, the primers were designed to amplify the full length of the human MT1G cDNA with an enzyme digest site of *Xba*I added to the 5′ end of the forward primer and an enzyme digest site of the *Bam*HI added to the 5′ end of the reverse primer. The forward primer was 5′-TAG TCT AGA ATG GAC CCC AAC TGC TCC -3′ and the reverse primer was 5′-TAT GGA TCC GGC GCA GCA GCT GCA CT -3′. MT1G cDNA was amplified using plasmid pACT2-MT1G as template. PCR conditions were as follows: 1 cycle at 94 ℃ for 2 minutes, 35 cycles with 30 seconds at 94 ℃ for denat-

uration, 45 seconds at 56 ℃ for annealing, 1 minute at 72 ℃ for extension and a final extension at 72 ℃ for 10 minutes. PCR products were identified with 1.5% agarose gel electrophoresis.

2.2.2 Construction and identification of recombinant eukaryotic expression vector pcDNA3.1(−)-MT1G

PCR products were purified by PCR purification kit and digested by *Xba* I and *Bam* HI. At the same time the plasmid pcDNA3.1(−) was also digested by *Xba* I and *Bam* HI. Later, the digested products of the 2 above mentioned were ligated by T4 DNA ligase for overnight at 16 ℃. The ligation products were transferred into competent cells of *E. coli* DH5a. Amp-resistant clones were selected and positive clones were verified by PCR. The positive clone containing MT1G cDNA fragment was named pcDNA3.1(−)-MT1G. The construction procedure of recombinant vector is shown in Figure 1.

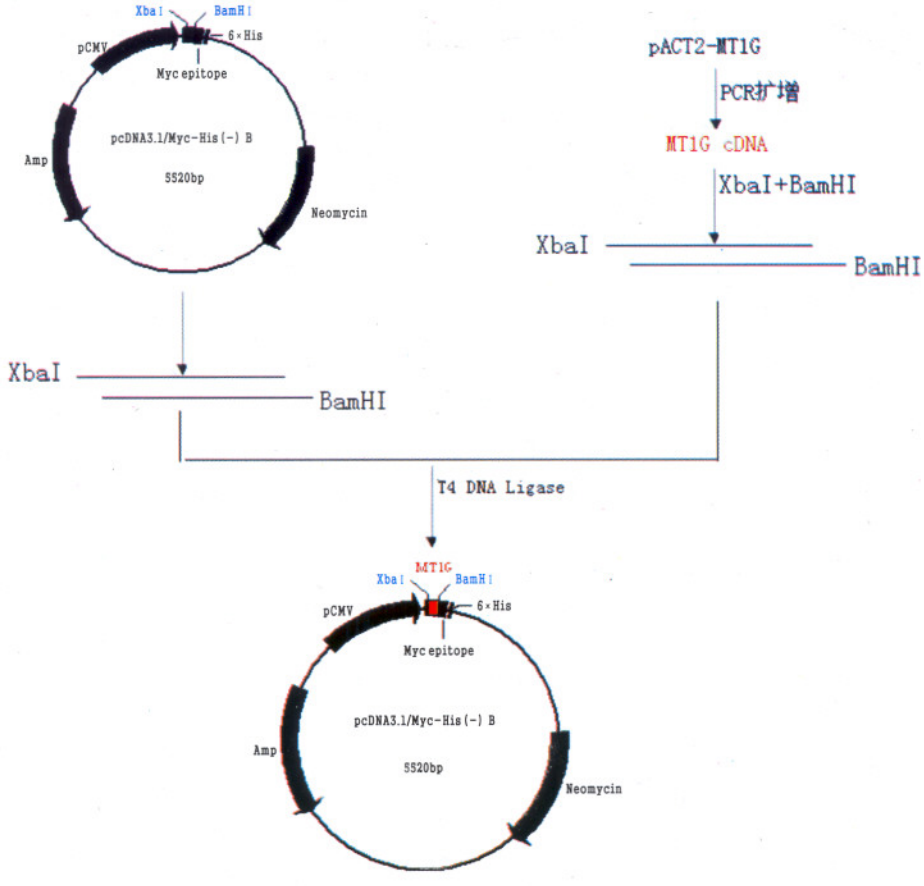

Figure 1. The construction procedure of recombinant vector pcDNA3.1(−)/Myc-His-MT1G

2.2.3 Cell tranfection and positive clone screening

According to the protocol of Lipofectamine™ 2000, plasmids pcDNA3.1(−)-MT1G were transfected into esophageal cancer cell EC9706. And pcDNA3.1(−) also was transfected transfected into EC9706 cells as negative control. Two groups cells were screened in the medium containing geneticin (G418) of 400 mg/mL for 48 hours. After two weeks' selection, positive clones were isolated and further expanded which were named EC9706-MT1G and EC9706-null cells respectively.

2.2.4 Identification of transfection

RT-PCR: Total RNA were extracted with TRIZOL reagent according to the manufacturer's instructions. Concentration and purity of RNA were measured by ultraviolet spectrophotometer. 0.5 μg RNA was used to synthesize cDNA, and PCR was performed as follows: 94 ℃ for 2 minutes;35 cycles of 94 ℃ for 30 seconds, 54 ℃ for 30 seconds, 72 ℃ for 1 minute; 72 ℃ for 5 minutes. The primers for MT1G were : 5′-TCG CTT

GGG AAC TCT AGT CTC-3′ (forward), and 5′-GCA AAG GGG TCA AGA TTG TAG -3′ (reverse), amplification fragment lengths were 309 bp. β-actin primers were 5′-CAT CCT GCG TCT GGA CCT-3′ (forward), and 5′-TCA GGA GGA GCA ATG ATC TTG-3′ (reverse), amplification fragment lengths were 480 bp. RT-PCR products were visualized by ethidium bromide-stained 1.5% agarose gel.

Westem blot: Cells were washed for 3 times with PBS and lysed in RIPA lysis buffer. Protein concentration in each lysate was detected by Bradford method. The protein were mixed with 5 × loading buffer and boiled for 5 minutes. Protein samples were resolved by SDS-PAGE by the same volume and transferred to 0.2 μm PVDF membrane. After blocking with 5% skim milk, proteins were incubated with rabbit anti-human His antibody for overnight at 4 ℃ and then with peroxidase-conjugaated secondary antibody for 1 hour. After washed for 5 times with PBST, the membrane was then stained using DAB kit.

3 Results

3.1 Identification of recombinant plasmid pcDNA3.1(−)-MT1G

Target gene fragment was obtained by PCR amplification from plasmid pACT2-MT1G containing human MT1G cDNA and inserted into pcDNA3.1(−) eukaryotic expression vector to construct pcDNA3.1(−)-MT1G recombinant plasmid. After digesting with double restriction endonuclease *Xba* I and *Bam* HI, around 5.4 kb and 192 bp fragments were obtained. The former is plasmid fragment digested and the latter was target gene fragment digested (Figure 2). Recombinant plasmid was sequenced, and the result was the same as MT1G cDNA sequence published in Genebank (Figure 3). This confirmed that eukaryotic expression vector pcDNA3.1(−)-MT1G was constructed successfully.

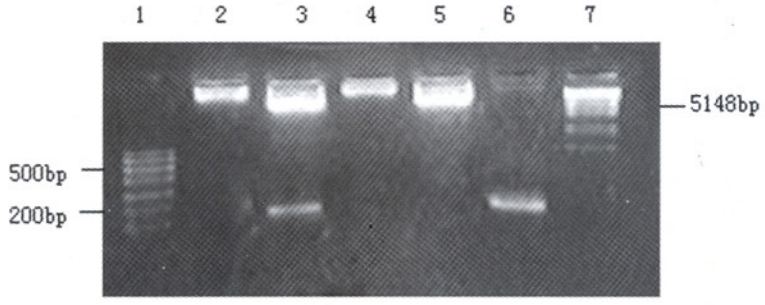

Figure 2. RCR analysis of recombinant plasmid pcDNA3.1(−)-MT1G
Lane 1: 100 bp ladder marker; Lane 2: pcDNA3.1(−)-MT1G; Lane 3: pcDNA3.1(−) -MT1G digested by *Xba* I and *Bam* HI; Lane 4: pcDNA3.1(−); Lane 5: pcDNA3.1(−) digested by *Xba* I and *Bam* HI; Lane 6: PCR product of MT1G Lane 7: λDNA/*Hind* Ⅲ + *Eco* RI marker

3.2 Expression of MT1G mRNA

RT-PCR products of the cell EC9706-MT1G yielded two expected fragments of 480 bp and 309 bp, corresponding to RT-PCR products of β-action and MT1G respectively. While RT-PCR products of the cell EC9706-null only yielded one fragment of 480 bp β-action and no target gene fragment of 309 bp(Figure 4).

3.3 Expression of MT1G fusion protein with his-tag

MT1G fusion protein with his-tag was detected in the cells EC9706-MT1G and not in cells EC9706-null(Figure 5). This showed stable transfection of *MT1G* gene in EC9706 cells was successful.

4 Discussion

At first, MTs were recognized to involved metal ion homeostasis and detoxification, protection against DNA damage, oxidative stress[7]. In the postgenomic era, it is becoming increasingly clear that MT fulfils different functions[8]. In recent years, more and more data have showed that MTs are associated with tumour cell proliferation and apoptosis, resistance to radiation or chemotherapy, patient survival and prognosis[9,10]. Although metallothionein expression has been implicated in carcinogenic evolution, current knowledge on the potential biological roles of its different isoforms in the various human cancers remains unclear. So far, researchers only found that different MT isoforms in humans possibly play different functional roles during development or under various physiological conditions[11]. Individual isoforms have unique functions[12]. Therefore, detailed studies focused

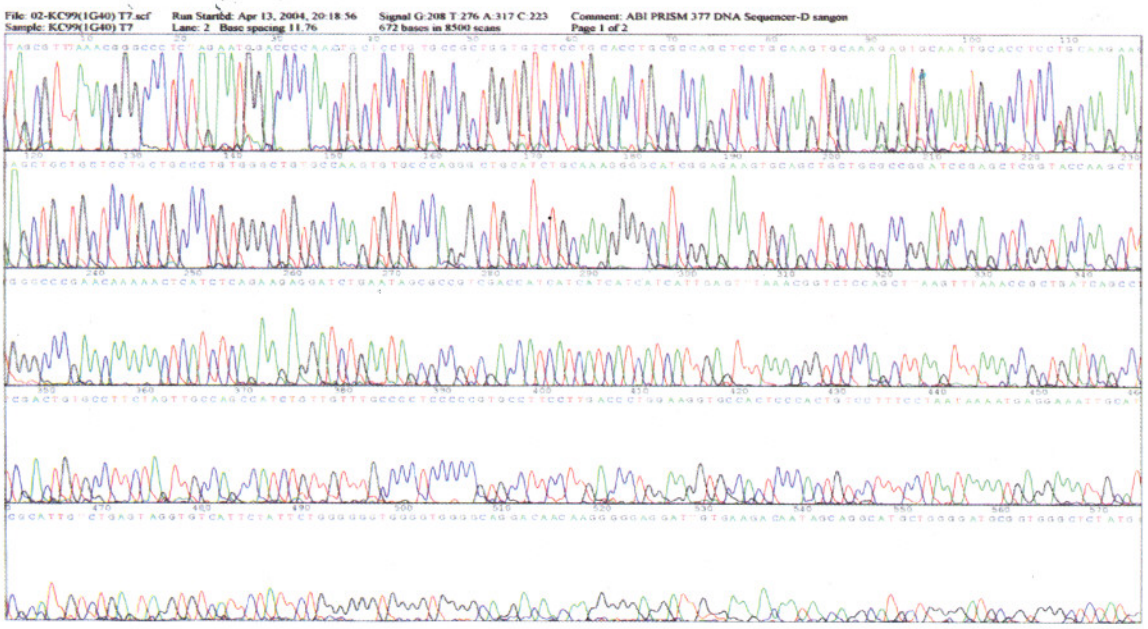

Figure 3. Recombinant eukaryotic expression vector pcDNA3.1(－)-MT1G sequence

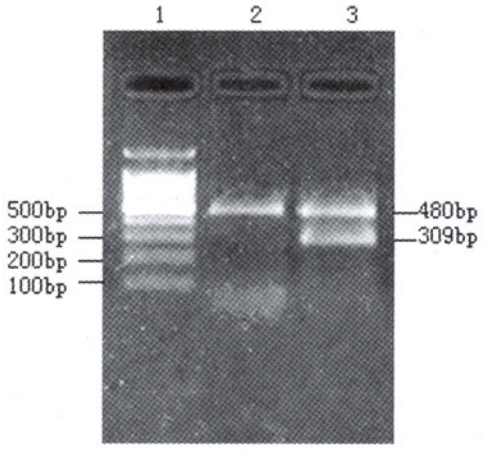

Figure **4**. Expression of MT1G mRNA detected by RT-PCR Lane 1: 100 bp ladder marker; Lane 2: EC9706-null; Lane 3: EC9706-MT1G

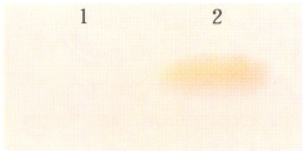

Figure 5. Expression of MT1G fusion protein with His-tag deteced by Western blot. Lane 1: EC9706-null; Lane 2: EC9706-MT1G

on the functional roles of different metallothionein isoforms could elucidate the role of this group of proteins in the carcinogenic process, delineating its possible clinical significance for the management of patients. In this study, we constructed the eukaryotic expression vector of MT1G and expressd it in human esophageal cancer cell EC9706 first.

Due to the high sequence homology of the MT isoforms, the generation of isoform-specific antibodies for MT was hindered[12]. The commercially available E9 antibody, which is used routinely for MT immunohistochemical studies, cannot distinguish different MT isoforms[13]. So, the eukaryotic expression plasmid pcDNA3.1/Myc-His(－) was chosen to encode two tag gene of myc and 6× His. The full MT1G cDNA was inserted into the multi clone site of the eukaryotic expression plasmid pcDNA3.1/Myc-His(－) and located closely at the upstream of two tag gene of myc and 6× Poly-histidines so as to construct recombinant eukaryotic expression vector of MT1G gene: pcDNA3.1/Myc-His(－)-MT1G. Recombinant plasmid was tranfected in esophageal cancer cell EC9706. Then expression of the MT1G fusion protein with 6× His tag was detected with specific anti-6× His antibody which could reflect expression level of MT1G protein indirectly. Although fusion protein was obtained, 6× His tag was very small and stable in physiological pH, folded structure and chemical properties of recombinant protein were not changed and the tag often has little effect on the function of the protein. In addition, pcDNA3.1/Myc-His(－) contains CMV promoter and SV40 ori reproduce

component and provides efficient, high-level expression in a wide range of mammalian cells. Neomycin resistance gene was used to selection of stable transfectants in mammalian cells.

In this study, eukaryotic expression plasmid of MT1G gene was constructed and transfected into human esophageal cancer cells EC9706 successfully. This layed a foundation for further study on the function and mechanism of MT1G in tumorenesis.

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