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Editors:

Radim Cerkal

Natálie Březinová Belcredi

Lenka Prokešová

Aneta Pilátová

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Optimization of multiplex RT-PCR for selected isoforms of metallothionein genes and influence of cisplatin on prostatic cell lines

Frantisek Petrlak[1], Veronika Smidova[1], Zbynek Splichal[1,2], Petr Michalek[1,2]
[1]Department of Chemistry and Biochemistry
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Central European Institute of Technology
Brno University of Technology
Purkynova 123, 621 00 Brno
CZECH REPUBLIC

petrlak.fanda@gmail.com

Abstract: This study deals with the issue of metallothioneins as potential tumor markers using the multiplex reverse transcription polymerase chain reaction (RT-PCR) method, for which specific primers were designed, with the subsequent sequencing of their products. The following step involved a study of expression using the PCR method for selected *MT1A*, *MT2A* and *MT3* genes after treating cell lines with cisplatin. Experiments were carried out on PNT1A, LNCaP and DU145 prostatic cell lines. Despite a very high similarity of the individual isoforms of metallothioneins, a triplex of three genes for two cell lines, PNT1A and DU145, was created. Upon a statistical evaluation of data obtained via the method of polymerase chain reaction in real time, it was found that, after the application of cisplatin, there was an increase in metallothionein expression.

Key Words: biomarkers, cisplatin, metallothioneins, PCR, prostatic cell lines

INTRODUCTION

Metallothioneins (MT) are proteins rich in amino acid cysteine which increases their affinity for metals. MT protects the cell from the toxic effects of heavy metals, DNA damage and oxidative stress. In addition, they play a role in cancer, preventing apoptosis and participating in tumor cell proliferation (Si and Lang 2018).

In humans, 16 localized genes on chromosomes have been identified that encode four isoforms of MT marked with numbers (*MT1* to *MT4*). Proteins of MT2A, MT3 and MT4 are encoded by a single gene located on chromosome 16. MT1 contains many (sub)isoforms. The known active (sub)isoforms of *MT1*gene are *MT1A*, *1B*, *1E*, *1F*, *1G*, *1H*, *1M* and *X*.

In this study MT were studied in connection with prostate cell lines. Detecting changes in the expression of individual MT isoforms could contribute to early tumor diagnosis and targeted therapy. It is believed that future studies of MT will reveal not only their functions in the pathogenesis of cancer, but also provide new insights into diagnosis and therapy (Cherian et al. 2003).

MTs also play a critical role in the treatment of cytostatics. They can bind cisplatin compounds and remove them from cells, which can lead to resistance. Monitoring the expression of MTs in tumor cells may be useful in selecting a treatment method (Bizoń et al. 2017).

MATERIAL AND METHODS

This study was divided into two experiments. The first experiment was based on designing primers for isoforms and (sub)isoforms using the PCRTiler v1.42 web software, then these primers were used for RT-PCR multiplex and optimization. In the second experiment, the selected cell lines were treated with cisplatin and after an incubation time of 24 h the expression of different isoforms of *MT* (*MT1A*, *MT2A* and *MT3*) in selected cell lines (PNT1A, DU145 and LNCaP) was measured.

Cell lines and cultivation

Prostate cell lines DU145, PNT1A and LNCaP were used for given experiments. Cultivation was performed in RPMI 1640 medium with 10% fetal bovine serum and a mixture of the antibiotics penicillin and streptomycin (200 U/l penicillin and 0.2 mg/l streptomycin). Cell lines were cultured in an incubator at 37 °C, 5% CO_2 and 70–90% humidity. Cells were maintained in an incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany). Cells were counted using Countess II FL (Thermo Fisher Scientific, Waltham, MA, USA).

The first cell line used was PNT1A. It is a primary culture obtained from normal adult prostatic epithelial cells of a 35-year-old male *post mortem*. Immortalisation was established by transfection with a plasmid containing SV40 (simian virus 40). The second cell line was human hypotriploid prostate cancer DU145. These cells have moderate metastatic potential and they are positive at the androgen receptors. The last tested cell line was LNCaP which are androgen-sensitive human prostate adenocarcinoma cells derived from metastasis from a 50-year-old. They are adherent epithelial cells growing in aggregates and as single cells.

Isolation and transcription

RNA isolation was performed according to the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) manufacturer's instructions. Synthesis of cDNA from all 4 cell lines was performed with a Transcriptor First Strand cDNA Kit (Roche, Basel, Switzerland) with a total yield of 2000 ng in a Mastercycler realplex^4 thermocycler (Eppendorf, Hamburg, Germany).

Multiplex RT-PCR

For better optimization, two PCR methods were combined, touchdown and multiplex. This increased the annealing specificity of the primers. The RT-PCR multiplex reaction mixture consisted of a 10 µl Luna® Universal One-Step RT-qPCR Kit, (NEB, Massachusetts, USA), 0.5 µl of forward and reverse primer (Sigma-Aldrich, St. Louis, Missouri, USA) and template DNA with a final volume of 20 µl. The PCR procedure is described in Table 1.

Table 1 Multiplex RT-PCR reaction conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	25 s	23
Annealing, Extension X= 65 °C	X = X - 0.3 °C	35 s	
Denaturation	95 °C	25 s	20
Annealing, Extension	58.1 °C	35 s	
Melting curve	60–95 °C	20 min	1
Cooling	4 °C	∞	∞

Agarose gels

All agarose gels were composed of 1 g of agarose dissolved in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA). The separation time was 90 min at 80 V. Agarose gels were visualized and photographed under 365 nm UV radiation on the Azure c600 from Azure Biosystems (Dublin, California, USA).

MTT assay

The viability was detected using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A 50% inhibitory concentration for cisplatin after 24 h was determined. Cisplatin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), diluted with 10% NaCl solution to a concentration of 1 mg/ml and dissolved at 80 °C for 5 min. After treatment, 10 µl of MTT (5 mg/ml in phosphate buffered saline) was added to the cells and incubated. After that, MTT containing medium was replaced by 100 µl of dimethyl sulfoxide and absorbance was determined at 570 nm using Infinite 200 PRO (Tecan, Männedorf, Switzerland).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Relative gene expression was calculated for *MT1A*, *MT2A* and *MT3*. GAPDH was used as a housekeeping gene. Luna® Universal One-Step RT-qPCR Kit was used as a master mix for quantitative PCR analysis; the analysis was performed using the Mastercycler realplex^4 (Eppendorf, Hamburg, Germany).

GraphPad Prism 8

Statistical analysis was performed using Student's t-test and the difference between groups was assessed as significant at $p < 0.05$. Statistics were performed from three biological replicates.

RESULTS AND DISCUSSION

The aim of first experiment was to design complementary primers to cDNA of individual isoforms and (sub)isoforms of MT. This was complicated by the high similarity between the sequences of the individual genes. Finally, primers were successfully designed. The products of primers were verified in agarose gel with following by optimization of the multiplex RT-PCR. Primers are shown in Table 2.

Table 2 Overview of primer design s for multiplex RT-PCR analysis

Name	Forward primers 5'–3'	Reverse primers 5'–3'	Amplicon size (bp)
MT1A	TCTGCAAAGGGGCATCAGAG	TGGGTCAGGGTTGTATGGAA	122
MT1B	GAACTCCAGGCTTGTCTTGG	GATGAGCCTTTGCAGACACA	187
MT1E	GCTTGTTCGTCTCACTGGTG	TTGCAGGAGGTGCATTTG	136
MT1F	GCTTCTCTCTTGGAAAGTCCAG	TTGCAGGAGGTGCATTTG	128
MT1G	CTAGTCTCGCCTCGGGTTGCA	CAGGAGCAGCAGCTCTTCTTGC	128/131
MT1H	CTGGGCTGTGCCAAGTGTG	ATGAGTCGGAGTTGTAGAAA	153
MT1M	AGCAGTCGCTCCATTTATCG	AGGAGCAGCAGCTCTTCTTG	157
MT1X	TCTCCTTGCCTCGAAATGGAC	TTGCAGGAGGTGCATTTG	104
MT2A	CTCGTCCCGGCTCTTTCTA	GAGTCGGGACAGGTTGCAC	101
MT3	TCGACATGGACCCTGAGACC	CACACTTCTCACACTCCGCA	141
MT4	ATGGACCCCAGGGAATGTGT	CTGAGCCTCCTTTGCAGATG	166

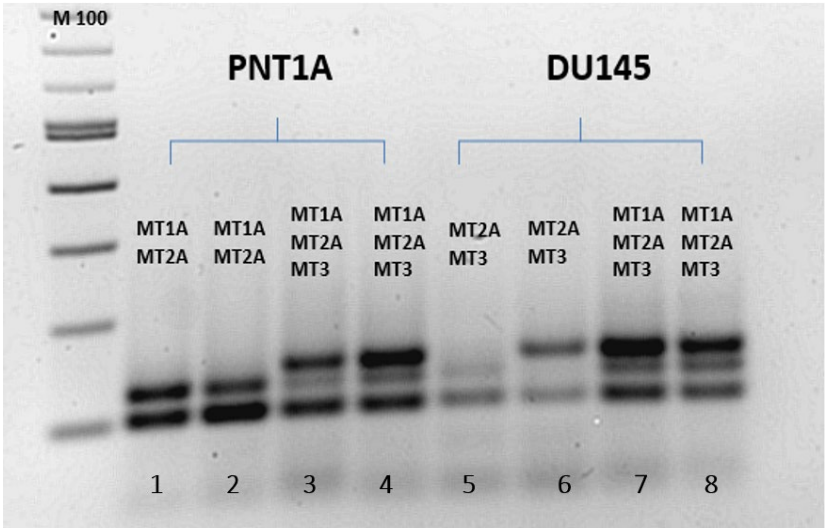
Optimization consisted of the correct annealing temperature and the amount of primers and cDNA. After testing all possible (sub)isoforms by multiplex RT-PCR, some duplexes were detected, but the most successful result is triplex in the last well of DU145 with *MT1A*, *MT2A* and *MT3*. The products and their semi-quantitative amounts can be identified in Figure 1. The best combination of amounts of primers and temperatures on Multiplex RT-PCR has opened new possibilities for further study of these genes.

The aim of the second experiment was to determine whether cisplatin affects the expression of genes in prostate cell lines. The dependence of cisplatin concentration on cell line vitality was measured using MTT assay. The result of this assay was the calculation of which concentration of cisplatin leads to 50% viability of cells (termed as IC_{50}). For the PNT1A and LNCaP lines IC_{50} was found to be 15 µg/ml and for the DU145 line 62.5 µg/ml. At this concentration, the cells were treated with cisplatin and cultured for 24 h prior to the next part of the experiment. RT-qPCR was performed on selected *MT1A*, *MT2A* and *MT3* genes after cisplatin treatment and subsequent comparison with control samples. The proposed multiplex primers were used to study this hypothesis. The results are shown in Figures 2–4.

As shown in Figure 2, the expression levels of *MT1A* in the non-tumor PNT1A line does not change significantly compared to the control. There is no statistically significant difference ($p \geq 0.05$). For the *MT1A* gene in the LNCaP line, a big increase in expression is observed over the control, where

there is a very statistically significant difference (*p ≤ 0.05). For the DU145 cell line (p ≥ 0.05), slightly increased level of *MT1A* is observed compared to control.

Figure 1 Multiplex PCR products after successful optimization



Legend: 2% agarose gel, staining by ethidium bromide (10 ng/ml), M means 100bp DNA Ladder

Figure 2 Effect of cisplatin on MT1A in prostate cell lines

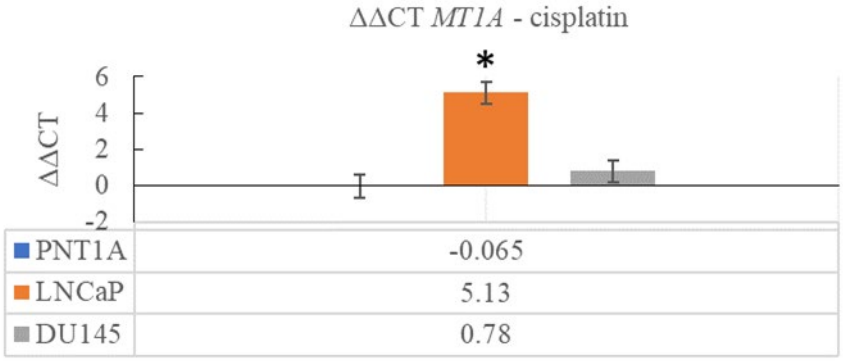
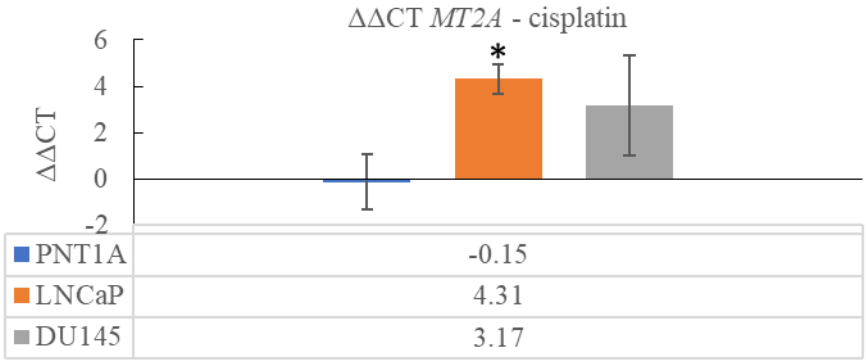


Figure 3 Effect of cisplatin on MT2A in prostate cell lines



As shown in Figure 3, the change in *MT2A* gene expression was slightly divergent from the control in the PNT1A line. These results were not significantly different from the control (p ≥ 0.05). In the LNCaP cell line, an increase in *MT2A* expression was observed in the untreated line, which was assessed as significant (*p ≤ 0.05). These results are confirmed by a study describing an increase in *MT2A* expression associated with resistance to chemical drugs (Kondo et al. 1995). The DU145 line shows a similar trend in *MT2A* gene expression over control. Expression of this gene in the DU145 line is statistically without significant differences from the control (p ≥ 0.05).

Figure 4 Effect of cisplatin on MT3 in prostate cell lines

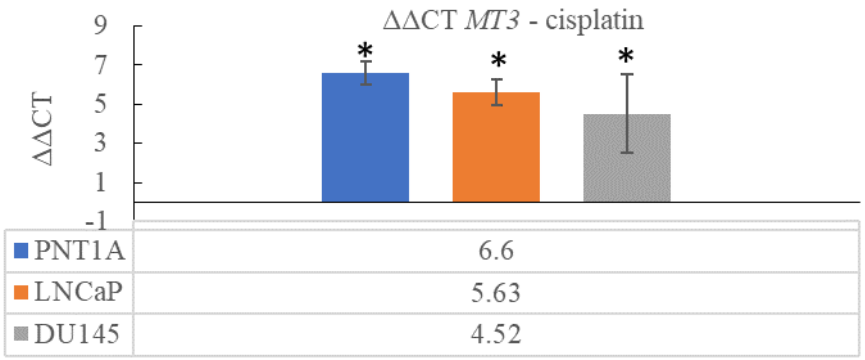


Figure 4 shows that the expression of the *MT3* gene in the PNT1A line is increased compared to the control. In this case, there is also a highly statistically significant difference ($*p \leq 0.01$) from the established control. Another result was determined in LNCaP cell line, where there is also a significant difference ($*p \leq 0.05$) compared to the *MT3* control, whose expression shows an increase over the untreated line. The last cell line to measure *MT3* expression was DU145 where expression level was also increased over the control. This cell line also showed a statistically significant difference from the control ($*p \leq 0.05$).

Dutta et al. (2002) also disclose *MT3* overexpression enhancing chemotherapeutic drug resistance in PC3 prostate cancer cells. Overexpression of *MT3* after cisplatin administration suggests that *MT3* may play a similar role to *MT1A* and *MT2A* in metal homeostasis in prostate cells (Garrett et al. 1999).

The results show that *MT* expression is increased after cisplatin application. This phenomenon has also been observed in several other publications. Cisplatin causes oxidative stress leading to cell apoptosis. However, *MT1A*, *MT2A* and probably *MT3* are also capable of capturing free radicals, even having the ability to bind cisplatin itself (Kelley et al. 1988, Brozovic et al. 2010).

CONCLUSION

In this study the multiplex touchdown RT-PCR method using more than one set of primers for various MT isoforms was successfully designed. As a result, *MT1A*, *MT2A* and *MT3* gene triplex was established in two cell lines, PNT1A and DU145. The object of the design and optimization of multiplex RT-PCR was to find a suitable diagnostic method that would be less expensive and, its implementation would be faster and easier compared to other diagnostic methods with the elimination of errors.

In the second experiment, after MTT assay and cultivation of the cells with cisplatin for 24 h RNA isolation and transcription into cDNA by reverse transcriptase was performed. Expression was measured by RT-qPCR. Statistical evaluation of the obtained data confirmed increased expression of *MT1A*, *MT2A* and *MT3* genes in cisplatin-treated cells. Increased expression was observed in the *MT1A* gene over the untreated control in the LNCaP line. This trend was also observed in the *MT2A* gene on the same cell line, which was a human prostate cancer cell derived from metastatic cells. LNCaP and DU145 tumor lines and non-tumor PNT1A lines have been shown to be overexpressed with *MT3*. Overexpression of *MT3* following cisplatin administration suggests that *MT3* may play a similar role to *MT1A* and *MT2A* in metal homeostasis in prostate cells. This overexpression may be due to the affinity of metallothionein to metals, or to the formation of a high concentration of oxidative stress caused by cisplatin in cell lines.

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