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The delayed effect of mustard gas on housekeeping gene expression in lung biopsy of chemical injuries

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ABSTRACT

Objective: Sulfur mustard (SM) was used as a chemical weapon in Iraq-Iran war. Exposed people have major complications in important organs such as pulmonary system. Some studies have shown that SM could affect the expression of endogenous genes and non-housekeeping genes, time dependently. To understand the accurate molecular mechanism of the delayed effect of SM, the identification of the gene expression pattern in these patients is essential. Hence, we have evaluated mRNA expression of four common housekeeping genes (ACTIN, PGK1, β2m, GAPDH) in SM-exposed and non-exposed (control) formalin-fixed, paraffin-embedded (FFPE) human lung tissues.

Method: Paraffin block of lung biopsy of SM-exposed people (11 cases) and people without exposure to SM as control group (9 cases) have been selected. The mRNA expression of four endogenous control genes has been evaluated by qRT-PCR. The stability value of each gene was calculated by different methods.

Result: It was found that ACTIN mRNA has the highest expression (30.26 ± 2.87) and PGK1 has the lowest standard deviation (SD) (30.885 ± 2.215) between pooled groups. The best correlation was between ACTIN and PGK1 expressions. The M value has shown that ACTIN and then PGK1 are the most stable housekeeping genes among. The results obtained from the GeNorm and NormFinder have indicated that the pair ACTIN- PGK1 is the most suitable choice for endogenous control genes.

Conclusion: ACTIN and PGK1 genes are stable in studied lung tissues and are the better than two other housekeeping genes. In addition, mustard gas does not affect their expression in long term.

1. Introduction

Sulfur mustard (SM) is known as a chemical weapon and has been used by Iraq military forces several times in Iraq-Iran war during 1980–1988 [1]. SM is an alkylating agent which alters the DNA structure and may cause formation of intra- or interstrand crosslinks which finally induce double strand breaks in DNA [2]. Onset of the signs and the symptoms of SM exposure is 30 min to 6 weeks after exposure. But, exposed people suffer from delayed toxic effects of SM for even more than 20 years after exposure [3]. The main problem of the chemical injured people is ocular and pulmonary complications as well as skin tissue involvements [4]. Despite numerous studies, the molecular mechanism of SM in chronic phase and curative treatment for its long-term respiratory complications has not been found yet [2,5]. Therefore, a molecular study to identify the precise mechanism of tissue damage is necessary to promote the diagnosis and treatment procedures.

Some studies have shown that SM has effect on gene expression in vitro and in vivo conditions. Steinritz et al. [6] showed that the GAPDH protein (Glyceraldehyde-3-Phosphate Dehydrogenase), a usual housekeeping gene, is up regulated when human keratinocyte cell line is exposed to SM. Also Vallet et al. [7] demonstrated that SM has a time dependent effect on gene expression in hairless mice. They showed that expression of IL-6, IL-1b, MIP-1aR and Cxcl2 genes have upregulated as early as 6 h from exposure to after sufficient time for wound repair (over 14 days). However, K1 mRNA level increased only 21 days after SM challenge.

Because any alteration in gene expression could be a potential source for clinical problems of SM exposed people, it is necessary to

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evaluate genes expression in these patients. To this end, molecular techniques such as quantitative real-time PCR (qPCR) are being used by researchers. qPCR is known as a gold-standard assay for measuring the gene expression and is used for confirmation of microarray data [8]. This method is commonly performed to achieve the pattern of target genes expression due to its high sensitivity, specificity and broad quantification range [9]. The internal controls are essential to ensure the accuracy and reliability of qPCR results and also to normalize the target gene with an endogenous gene. Hence, housekeeping genes are indispensable to molecular studies. They should have stable expression in all tissue and cells of organism and do not affected by the external signal or cell cycle stages [10].

Given that sulfur mustard could alter the DNA structure and gene expression, in this study, which is a preliminary segment, we have tried to evaluate mRNA expression level of different housekeeping genes in lung tissue of chemical injuries after 25–30 years after SM exposure. The studied housekeeping genes include beta actin(β-actin) is a major part of the contractile apparatus, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the main enzymes of glycolysis pathway,beta-2-microglobulin (β2m)) is an extracellular part of MHC I complex, and phosphoglycerate kinase 1 (PGK1) catalyzes reversible transfer of a phosphate group from 1,3-bisphosphoglycerate. Finally, the most stable endogenous gene in SM exposed people has been chosen by comparing the threshold cycle (CT) mean, dispersion of studied genes expression, and calculating the gene expression stability measure (M value).

2. Material and method

2.1. Ethical statement

This study is part of a comprehensive research which has been approved in Immunoregulation Research Center, Shahed University and Research Ethics Committees of Shahed University and Medical Faculty of Trabiat Modares University.

2.2. Sample collection

This study has been performed on paraffin blocks of lung biopsy. All samples have been collected from archived blocks of department of pathology. These blocks have been obtained by surgical resection at general hospitals in Tehran, Iran during 2005–2011. The samples were used with a code without any name or other individual characteristic. The exposed group was lung biopsy of people with documented mustard gas exposure in Iraq-Iran war (1980–1988), which had delayed pulmonary complications (n = 11). SM exposed people were excluded if they had systemic or local diseases affecting on the study like acute and chronic infection, autoimmune disease, history of other toxic gases exposure and job pollutions. The control group (n = 9) was lung biopsy of patients that they had undergone surgery for diagnosis of their pulmonary diseases. The main inclusion criteria for control group was; to have normal histology block. These patients should not had history of exposure to mustard gas or other toxic gases and occupational pollutions, chronic disorders such as systemic or pulmonary inflammation diseases, systemic or local acute and chronic infection, autoimmune disease, and asthma. The hematoxylin and eosin (H & E) stain slides of control group evaluated by a pathologist. Blocks with normal histology were confirmed and were chosen for study. Both groups were men with age 30–60 years and were not smoker and had no history of addiction to opiates and alcohol. More data about samples is shown in Table 1.

Table 1
Histopathological characteristics of the exposed and control groups.

	Exposed (n = 11)	Control (n = 9)
Age (y)	42.7(33–61)	51.1(24–67)
Sex	Male	Male
Diagnosis		
Constructive bronchiolitis	7	0
Chronic bronchitis	2	0
Bronchiectasis	1	1
Anthracosis	0	2
Benign tumor	1	1
Malignant tumor	0	4
Sequestration chronic inflammation	0	1

2.3. RNA isolation and DNase treatment

Total RNA was extracted from collected tissue specimens by RNeasy FFPE Kit (Qiagene- Germany) and the manufacturer's protocol was carried out with a little modify. Firstly, four 10 μm sections were cut from each specimen and were deparaffinized in xylene (Merk-Germany) at 56 °C for 2 × 30 min with 400 rpm agitation. The residual xylene was washed by ethanol 96% (Merk-sigma). After drying in the air, the cell membrane was disrupted by heating at 56 °C for 3 h with proteinase K. In the following, proteinase K was inactivated with incubation of the pervious step supernatant at 80 °C for 15 min. DNase treatment was done by mixing of DNase Booster and DNase I and its time was increased to 20 min. Then, RBC and RPE buffers were added exactly according to the manufacturer's protocol, respectively. The extracted RNA was washed from RNeasy® MinElute® Spin Columns by RNase free water. The concentration and purity of RNA were measured via the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The quality of RNA sample was determined according to OD 260/280 ratio and the sample was discarded when OD 260/280 ratio was less than 1.8. The common method for quality measurement of the extracted RNA is determination of agilent RNA integrity number (RIN). But, it has been shown that the RIN values from degraded FFPE samples are not a sensitive measure of RNA quality. Instead, the investigators have found that the mean RNA fragment size is useful as a determinant of RNA quality for the extracted RNA from FFPE tissues [11]. Therefore we have used the percentage of RNA fragments with more than 200 nucleotides (DV200) to evaluate the quality of RNA. DV200 metric analysis was performed by Macrogen Co. (Korea) and the RNA samples with DV200 > 30% were accepted.

Our pilot study, which its results are not presented here, revealed that despite DNase treatment was done in RNA isolation step, genomic DNA contamination has remained yet. Therefore extra DNase treatment (fermentase protocol) was carried out on eluted RNA. For this aim, 1 μl buffer and 1 μl DNase was mixed for each sample. Then 1 μg extracted RNA was added and incubated at 37 °C for 45 min and 1 μl EDTA was added. After that, incubation was done at 65 °C for 10 min.

2.4. Reverse transcription

Reverse transcription of the pure RNA was performed by a high capacity cDNA reverse transcription kit (ABI-USA). According to the manufacturer's instructions, to prepare 2X reverse transcription master mix, 2 μl 10X RT buffer, 0.8 μl 25X dNTP Mix (100 mM), 2 μl 10X RT random primers and 1 μl MultiScribe™ Reverse Transcriptase were mixed together. For each sample, 10 μl master mix was added to 1 μg extracted RNA (~10 μl of RNA) and final volume per reaction was increased to 20 μl by nuclease-free H_2O. The reactions took place at 25 °C for 10 min, followed by 37 °C for 120 min, and 85 °C for 10 min in a thermal cycler T100 (BioRad, USA).

2.5. Real time PCR TEST

The mRNA expression of four endogenous control genes; ACTB (NM_001101.3), β2m(NM-004048), GAPDH (NM-002046) and PGK1(NM_000291.3), were determined by comparing their expression in different samples. Their primers were manually designed according