A Comparative Study of Metallothionein Gene Expression in Peripheral Lymphocytes and Blood Cadmium Level among Die Casting Male Workers

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Abstract

Cadmium were found in the die casting factory as by product of zinc alloy that used in the manufacturing of die cast. Metallothionein (MT) a carrier protein plays an important role in the detoxification process of cadmium in human. The usefulness of MT gene expression in peripheral blood lymphocytes (PBLs) as a biomarker of cadmium exposure and susceptibility could be determine by reverse transcriptase–polymerase chain reaction (RT-PCR). 41 male workers from die casting factory were involved in the cross-sectional comparative study and were divided into exposed and comparative group. MT gene expressions were found to proportionates increase with blood cadmium (BCd) levels. MT basal expression levels were significantly correlated with the BCd levels with r-value 0.616 for exposed group and 0.639 for comparative group. MT induction expression level were significant correlated with BCd level ($r = 0.188$ for exposed group, $r = 0.342$ for comparative group). This suggested that MT gene expression in PBLs can be used as a biomarker of susceptibility to cadmium.

Keywords: Metallothionein gene expressions, Blood cadmium, Reverse transcriptase-polymerase chain reaction, Die casting, Male workers

1. Introduction

Casting is a manufacturing process by which a liquid material is usually poured into a mold, which contains a hollow cavity of the desired shapes, then allowed to solidify. The casting process is subdivided into two distinct subgroups: expandable and non-expandable. Die casting is one of the methods in non-expandable mold casting that the mold need not be reformed after each production cycle. It is the process of forcing molten metal under high pressure into mold cavities from non ferrous metals.

Zinc alloy is the major contain in the die casting manufacturing company. Cadmium is one of the heavy metal that contain in the zinc alloy. Cadmium is isolated from zinc alloy by vacuum distillation, or cadmium sulfate is precipitated out of the electrolysis solution in the die casting manufacturing process. Die casting workers were exposed to emitted cadmium in the workplace.

Emitted Cadmium is an extremely toxic metal, particularly where any of it is being processed or smelted (16).The permissible exposure limit (PEL) is five micrograms per cubic meter of air (5 $\mu g/m^3$), calculated as an eight-hour time-weighted average exposure (TWA), overexposures may occur even in situations where trace quantities of cadmium are found in a smelter dust (2).

1.1 Health Effect of cadmium to human body

Cadmium is known as worldwide pollutants. With long half life (10-30 years) and in human body, it poses several adverse health effects (4) (15). Cadmium causes multi organ health effects at molecular, cellular and organ function. Studies have associated chronic occupational exposure to cadmium fumes and dusts with increased risk of chronic obstructive lung disease and emphysema (8). Other respiratory effects of chronic occupational exposure to cadmium include chronic rhinitis, destruction of the olfactory epithelium with subsequent anosmia as well as the development of bronchitis (1) (17). Cadmium exposure also causes renal dysfunction and resorption of cadmium at the anterior part of vertebrate (itai-itai disease) (12). In addition, cadmium has the ability to cause mutation, DNA damage in the form of strand break, disruption the synthesis of Nucleic Acid and protein, DNA mismatch and also expression of the Metallothionein(MT) gene.(6)

1.2 Metallothionein gene expression

Metallothionein gene has its role in the detoxification of toxic metal especially cadmium. The expression of the MT suggesting an indicator of both cadmium exposure and of cadmium caused kidney dysfunction (10). MT is a family of stress proteins with a high content of cysteine and divalent metal. MT genes are ubiquitously expressed in many tissues of basal levels, and their expressions are induced readily by many factors, especially by metal ions such as cadmium, zinc, and copper (9). Studies on the metabolism of cadmium have proven the role of MTs in the absorption, transport, and excretion of heavy metals (5). Thus, MT expression could be specific biomarkers for cadmium exposure in industrial.

MT is synthesized and bind with cadmium in liver on cadmium induction. Cadmium were then entered the hematology system and blood were filtered through the kidney glomerular membrane. In tubular cells, cadmium MT degraded and free cadmium that left in the blood stream will still exert adverse effects because it exceeds the synthetic capacity of tubular cells. (7),(9)(11)(12)(13).

The validity of MT expression in peripheral lymphocytes (PBLs) as a biomarker of cadmium exposure was measured in messenger RNA (mRNA) level in PBLs from cadmium exposed workers using reverse transcriptase–polymerase chain reaction (RT-PCR). Hence, the relationship between blood cadmium and MT gene expression could be determined.

2. Materials and Methods

2.1 Study Design and Study Population

A cross-sectional study involving 41 die casting workers was conducted from 6 May 2009 until 7 October 2009 in a die casting company in Selangor, Malaysia using stratified random sampling methods.

2.2 Socio demographic data collection

Each subject was requested and facilitated by trained interviewer to answer a questionnaire. Personal data, such as incomes, education level, life style including smoking and drinking habits, working experience, and also health status were asked in the questionnaire section. Blood sample were collected in the same day.

2.3 Biological sample analysis

A total 10 ml of fresh blood were drawn from the worker's cubital vein into a lithium heparin vaccutainer. 2ml of blood were used to analyze blood cadmium (BCd) to estimate the occupational uptake exposure of cadmium

using Graphite Furnace Atomic Absorption Spectrometry (GFAAS) by National Institute of Occupational Safety and Health (NIOSH), Malaysia.. Lymphocytes used for MT mRNA level measurement were isolated from the whole blood by using Ficoll-Paque(GE Health Care) and Accuspin tubes (Sigma-Aldrich).

2.4 Culture and treatment of PBLs

PBLs were isolated using Accuspin tubes and Ficoll-Paque from 8 mL of freshly collected vein blood. 15ml of Ficoll-Paque was pipetted into upper chamber of Accuspin tubes and was centrifuged for 1 minute. Whole blood was poured to the Accuspin tubes into the upper chamber of each Accuspin tubes. Lymphocytes were collected at the interface after centrifugation at 2000 rpm for 30 minutes. Lymphocytes were washed twice in phosphate-buffered saline and once in RPMI 1640 (Gibco). Isolated PBLs were then divided into 2 aliquots and incubated in 48 well cell culture plate containing RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum and 2% penicillin-streptomycin. Cadmium chloride (CdCl2) was added to 1 aliquot to the final concentration of 10 µM from a stock solution, and the same volume of ultra pure water (UPH2O) was added to another aliquot as control. After culture for 6 hours, cells were harvested and total RNA isolated.

2.5 RNA isolation and RT-PCR

Total RNA was isolated from harvested PBLs using QIAamp RNA Blood Mini kit (Qiagen) according to manufacturer's direction. Quantification and integrity of RNA were measured to check the concentration and total of RNA. Absorbences at 260 and 280 nm were measured using formaldehyde agarose gel electrophoresis.

MT mRNA level was measured by using semiquantitative RT-PCR. RT-PCR was performed in a 50 µl reaction mixture containing 10 µl of 5 x buffers, 2 µl of dNTP mix, 0.4 µl of ß-actin primer and 1 µl of MTII primer. (QIAGEN One-step RT-PCR kit). The following RT-PCR cycle was used: Reverse transcriptase in 50°C for 30 minutes, Initial PCR activation step in 95°C for 15 minutes, Denaturing process in 94°C for 30 seconds, Annealing temperature is 60°C for 1 minute and extension for 1 minute in 72°C. These cycles were repeated 30 times. PCR products (15µl) were electrophoresed through 1.3% agarose gel. Gel was stained in ethidium bromide for 10 seconds followed by 10 minutes of destaining. Results were photographed under UV illumination and subjected to densitometer analysis using Image J software. To normalize the difference in efficiency of reverse transcription and cDNA amplification, MT mRNA levels were calculated using the formula below: (density of MTII/198)/ (density of ß-actin/469). MT mRNA level of PBLs cultured with saline represented the basal expression; while MT mRNA level of PBLs treated with CdCl2 represented the MT induction expression.

2.6 PCR primers

Primers specific for MTII and ß-actin were referred from ref. (9) studies. Primers used to amplify MTII (5) were 5'- TCTTCAGCACGCCATGGATC-3' and 5'-CGGATGTCCACGTCACACTT-3'. They yielded a fragment corresponding to 198 bp coding region of MTII gene. Primers for ß-actin (9) were 5'-CGGATGTCCACGTCACAC- TT-3' and 5'-GTTGCTATCCAGGCT- GTGCT-3'. They amplified a 469 bp of ß-actin gene.

2.7 Statistical methods

The database was constructed in a computer using SPSS version 17. Independent T-test was used to analysis the difference of the blood cadmium between exposed and comparative group. The efficiency of MT basal expression and MT induction expression was performed using independent T-test as well. Relationship between MT gene expression and blood cadmium were determine using simple linear regression.

2.8 Ethical considerations

The ethics committee of Faculty Medicine and Health Sciences, University Putra Malaysia approved this study.

3. Results

3.1 Descriptive univariate analysis

3.1.1 Socio demographic data

Mean age for exposed group were 30.32±6.74 and 29±5.89 for comparative group while mean BMI for exposed group were 21.65±2.65 and 23.13±5.76. Statistic analysis showed that there were no significant different between exposed and comparative group in age ($t=0.525$, $p>0.01$) as well as body mass index ($t=0.457$, $p>0.01$).

3.1.2 Blood Cadmium concentration

The mean blood cadmium concentration in exposed group were 0.65±0.11µg/L while 0.31±0.19µg/L in comparative group. There was a significant different of of blood cadmium (BCd) between exposed and comparative group and exposed group had higher level than comparative group with $t = 6.927$, $p\leq0.01$ (Table 1).

3.1.3 RT-PCR analysis

A test was conducted for the sensitivity and specificity of the RT-PCR method. MT-II and ß-actin were amplified in the same reaction tube, enabling subsequent standardization of MT-II quantification. This co-amplification balances the difference of the amount of total RNA used and the difference of efficiency of transcription between reactions. We found that this method can detect MT expression using as little as 0.1 mg of total RNA, and there is no interference between the 2 sets of primers.

The mean MT basal expression was 1.89±0.32 for exposed group and 1.69±0.29 for comparative group while MT induction expression was 2.14±0.41 for exposed group and 2.10±0.49 for comparative group. To investigate which form of expression, ie, basal expression or induced expression, of MT in PBLs will better reflect exposure, we measured both basal and induced MT expression in this study (Table 2). Results show that MT basal expression within exposed and comparative group ($t=1.932$, $p<0.1$) is more reflect than MT induced expression ($t=0.342$, $p>0.1$).

3.2 Bivariate analysis

3.2.1 Relationship between sociodemographic and blood cadmium

There were no significant correlation between age and blood cadmium where $r=0.088$, $p>0.1$ similar for correlation between smoking status and blood cadmium ($r=0.105$, $p>0.1$).

3.2.2 Relationship between MT Gene Expression and Blood Cadmium

To further elucidate the relationship between MT expression and internal dose index, blood cadmium (BCd) was compared between MT gene expressions. It shows a statistically significant increase in both MT basal and induction expression, in relation to BCd. There is a significant correlation between blood cadmium and MT basal expression where $r = 0.616$ $p<0.001$ for exposed group and $r = 0.689$ $p<0.005$ for comparative group. For correlation between MT induction expression and blood cadmium, it show only low correlation and it is not significant result ($r =0.188$ $p=0.368$ for exposed group and $r = 0.342$ $p=0.195$ for comparative group).

4. Discussions

4.1 Respondent Background Data

Forty one respondents participated in this study and categorized into two different groups exposed and comparative. Twenty five workers who work in die casting process were selected as the exposed group (stamping, tumbling, shot blast and flatness). Sixteen workers who are working except in the process listed above were selected as control group. All studied subjects were men between the ages of 21-34 years.

4.2 Metallothionein gene expression

MT mRNA levels in PBLs are related to cadmium exposure, suggesting this measurement could be an indicator of cadmim exposure (9). The reason MT expression is involved in this research is MT induced in human tissues when cadmium is exposed and it also play a role in detoxification of cadmium. Expression of MT gene showed that organism is exposed with cadmium and has adverse effects of cadmium. Most cadmium in blood is found in blood cells, and PBLs provide a good source for measuring MT synthesis.

Ref. (5) experiment that MT basal expression in cadmium exposed workers is significantly higher than non exposed workers. In Ref. (15) studies, in vitro induced MT mRNA levels in PBLs is significantly correlated with cadmium exposure but not MT basal expression. In the previous studies, there is some argument whether basal or induced MT mRNA levels better reflect the cadmium exposure. Hence, in this research, both basal and induced expression was measured. The research indicate basal expression is significantly elevated in exposed group compared with the comparative group ($t=1.932$, $p<0.05$). Induced expression is not significant correlation within exposed and comparative group ($t=0.342$, $p>0.05$). Further analysis showed that MT basal expression is significantly correlated with blood cadmium ($r=0.616$, $p<0.001$ for exposed group and $r = 0.689$, $p<0.005$ for comparative group). Due to cadmium in the blood influences the MT mRNA levels in PBLs, since blood provides the microenvironment in which PBLs exist.

4.3 Blood Cadmium and Associated of Age and Smoking Status

Cadmium concentration is strongly age related, and usually it reaches a plateau at 50 years of age, consonant with an age-related degeneration of kidney reabsorption function (14). Smoking increase whole body cadmium level including blood, urine and renal cortex, (3) since tobacco in cigarettes contain cadmium. Age and smoking status and its correlation with blood cadmium level also included in this research. Particularly, it shows not significant correlation in the fact of this research analysis. One explanation is the age range is too narrow for the

general age effects to be seen (9) as the age group of the workers in the organization who were participated in this research are between 20 and 30 for expose group, and the workers who is over 30 normally were on the control group where exposure were minima. Actually, blood cadmium level in smokers is relatively higher than non smokers although the difference is not statistically significant. It is just that more than two third of the workers in this organization is smokers influence the peculiar result.

5. Conclusion

In conclusion, this study showed that MT basal expression level in PBLs is closely related to cadmium exposure. Hence, study indicated that MT basal expression in PBLs as biomarkers of cadmium exposure. Further studies should be done for environment population with exposure of cadmium in their residential area.

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Table 1. Blood cadmium between exposed and comparative group

Group	n	Blood Cadmium	t	p-value
Exposed	25	0.65±0.11	6.927	0.004
Comparative	16	0.31±0.19		

Table 2. MT gene expression between exposed and comparative group

Group	n	MT basal expression	MT induction expression
Exposed	25	1.89±0.32	2.14±0.41
Comparative	16	1.69±0.29	2.10±0.49

Table 3. MT basal expression between exposed and comparative group

Group	n	MT basal expression	t	p-value
Exposed	25	1.89±0.32	1.932	0.041
Comparative	16	1.69±0.29		

Table 4. MT induction expression between exposed and comparative group

Group	n	MT induction expression	t	p-value
Exposed	25	2.14±0.41	0.342	0.734
Comparative	16	2.10±0.49		

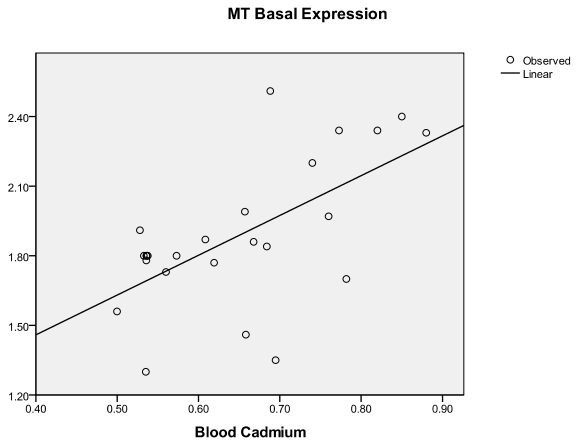

Figure 1. Regression Analysis for MT basal expression and blood cadmium for exposed group

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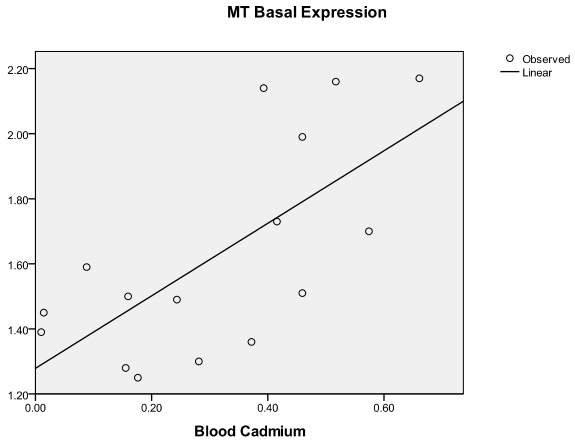

Figure 2. Regression analysis for MT basal expression and blood cadmium for comparative group

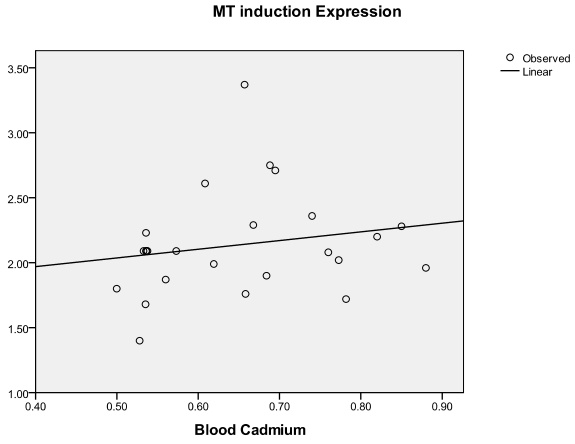

Figure 3. Regression analysis for MT induction expression and blood cadmium for exposed group

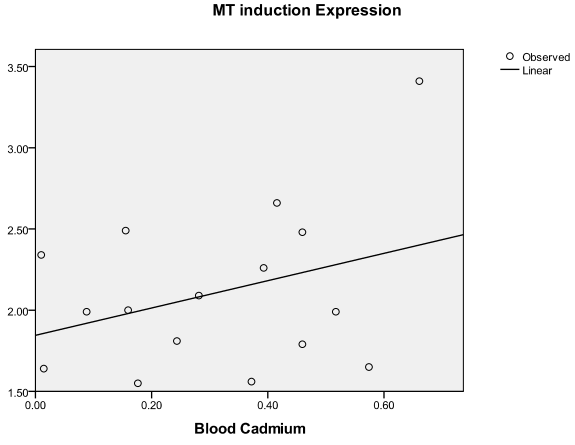

Figure 4. Regression analysis for MT induction expression and blood cadmium for comparative group

Figure 1-4 showed Regression analysis between MT gene expression and Blood Cadmium. It showed significantly correlate with r = 0.616 for exposed group and 0.689 for comparative group for MT basal expression while r =0.188 for exposed group, r=0.342 for comparative group for MT induction expression.

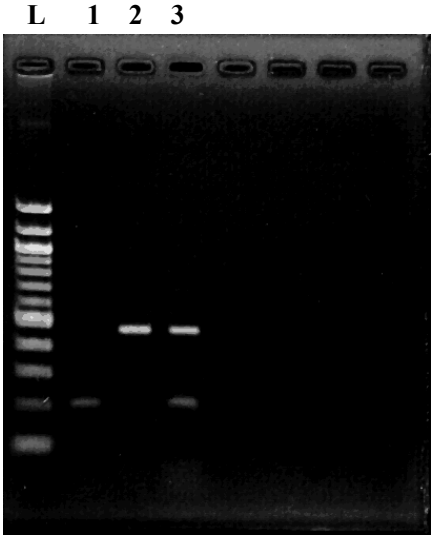

Figure 5. Amplification of MTII and ß-actin. L represented the 100bp DNA ladder (New England Biolabs); Lane 1 represented the MT II primer, Lane 2 represented the ß-actin primer whereas Lane 3 represented the duplex amplification within 2 primers.

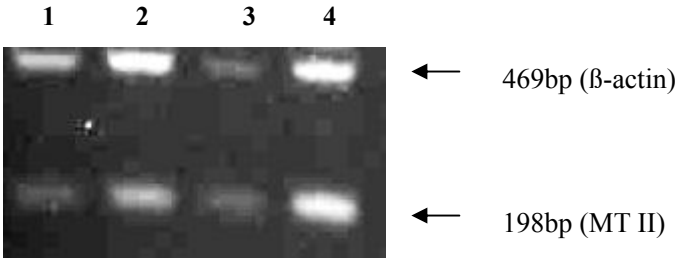

Figure 6. Representative result of a RT-PCR of MTII and ß-actin. This figure showed 2 subjects of respondents. MT mRNA levels in PBLs cultured with saline represented the MT basal expression (lane 1 and 3), whereas MT mRNA level in PBLs treated with $CdCl_2$ represented the MT induction expression (lane 2 and 4)

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