Research Article

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The effects of metallothionein 2A polymorphism on placental cadmium accumulation: is metallothionein a modifiying factor in transfer of micronutrients to the fetus?

Deniz Tekin,* Zeliha Kayaaltı, Vugar Aliyev and Tülin Söylemezoğlu

ABSTRACT: Metallothionein affects the metabolism, transport and storage of micronutrients such as zinc, copper and iron, and the detoxification of heavy metals, especially cadmium. Cd is a common, highly toxic environmental pollutant that accumulates in human placenta, elevated concentrations of which are associated with impaired zinc transfer to the fetus. This prospective study investigated the effects of metallothionein 2A (MT2A) –5 A/G single nucleotide polymorphism on the accumulation of Cd in human placenta and micronutrient transfer to the fetus in 95 pregnant women and their newborns. Venous blood from the mother was collected to investigate Cd, Zn, Cu, Fe levels and MT2A polymorphism. Cord blood from the neonate and placenta was collected for metal levels. MT2A polymorphism was determined by the standard PCR-restriction fragment length polymorphism technique. Metal levels were analyzed by Atomic Absorption Spectrometry (AAS). Maternal blood Cd levels were statistically higher for mothers with a heterozygote genotype compared with a homozygote genotype ($P < 0.05$). In contrast, placental Cd levels were significantly higher in mothers with a homozygote rather than a heterozygote genotype ($P < 0.05$). No difference existed in cord blood Cd, Zn and Cu levels. However, cord blood Fe levels of newborns with heterozygote genotype mothers were higher than in others. Placental Cd levels of heterozygote genotype mothers were negatively associated with Zn in cord blood. Cd exposure at environmental levels may be associated with alteration of the umbilical cord micronutrient levels for newborns with mothers of a heterozygote genotype. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: cadmium; metallothionein 2A; placenta; micronutrients; polymorphism

INTRODUCTION

Cadmium is a common, highly toxic environmental pollutant. Humans can be exposed to cadmium through tobacco smoking, their diet, drinking water and inhalation from the air. Another major source of cadmium is occupational exposure. Cadmium is also used in various industrial products such as electrodes in nickel–cadmium batteries, pigments (cadmium-yellow), coatings for machinery parts and alloys and control rods for nuclear reactors. Cadmium has a long biological half-life of 15–30 years, mainly due to its low rate of excretion from the body, and it accumulates over time in bones, kidneys and the liver as well as the reproductive organs, including the placenta (Henson and Anderson, 2000; Zadorozhnaja *et al.*, 2000).

Exposure to cadmium has particularly serious consequences for the mother and her developing fetus. Experimental animal studies show that placental Cd accumulation may adversely affect the fetus indirectly via impaired transfer of essential micronutrients (Webb and Samarawickrama, 1981; Maitani and Suzuki, 1986; Piasek *et al.*, 1996). Studies of the effects of Cd exposure during pregnancy in rats have found decreased fetus numbers (Baranski *et al.*, 1982; Samarawickrama and Webb, 1981) and fetal death (Ferm, 1971; Rohrer *et al.*, 1979; Levin and Miller, 1980). In addition, several studies have shown that neonates, even if born safely and without any apparent disabilities, are likely to have retarded growth (Rohrer *et al.*, 1979; Levin and Miller, 1980). Furthermore, Cd exposure during the gestational period significantly affects growth of offspring (Baranski *et al.*, 1983; Baranski, 1986). There is growing evidence that the fetotoxic effects of Cd may be mediated by altered zinc and copper metabolism (Sasser *et al.*, 1985). Human placenta perfused with high concentrations of Cd impairs the transfer of Zn from the maternal to fetal circulation (Wier *et al.*, 1990). Although the exact mechanism behind an interaction of Cd and Zn has not been understood, several studies have suggested that the accumulation of Cd in placenta induces the synthesis of the metal binding protein metallothionein (MT), which could lead to Zn retention in the placenta with a reduced flux of maternal Zn to the developing embryo (Kuhnert *et al.*, 1987; Ronco *et al.*, 2006). This is particularly important during pregnancy, when the developing fetus is vulnerable to inappropriate micronutrient status. Also, it is possible that the Cd-related malformations observed in rodents are due to a

*Correspondence to: D. Tekin, Ankara University, Institute of Forensic Sciences, Dikimevi, 06590, Ankara, Turkey.
E-mail: drdeniztekin@yahoo.com

Ankara University, Institute of Forensic Sciences, Dikimevi, 06590, Ankara, Turkey

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reduced delivery of maternal Zn to the embryonal compartment (Fernandez *et al.*, 2003). Infants of pregnant women exposed to environmental cadmium may experience adverse perinatal effects, such as decreased fetal growth and birth weight, congenital malformations and preterm birth (Goyer, 1990; Fréry *et al.*, 1993). These alterations are presumed to be mediated by metallothionein (a Cd-binding protein), which is the most important factor regulating the biological effects of cadmium. MTs are low-molecular-weight, cysteine-rich proteins. Because of their rich thiol groups, MTs bind to the biologically essential metals like Zn and Cu and perform these metals' homeostatic regulations (Sato and Bremner, 1993); they also absorb the heavy metals such as Cd and assist with their transportation and extraction (Nordberg and Nordberg, 2000). MTs detoxify heavy metals due to their binding capacity and protect the cells and tissues from the toxic effects of metals (Kondo *et al.*, 1995). Besides being a free radical scavenger, MTs are involved in multiple cellular functions involving immune response, genotoxicity and carcinogenicity (Nordberg, 1998). Induction of MT in the placenta occurs during gestation and has been suggested to prevent transfer of cadmium and regulate the transportation of zinc and copper from mother to fetus (Wade *et al.*, 1986; Goyer and Cherian, 1992; Ronco *et al.*, 2006; Sorkun *et al.*, 2007). However, the exact role of placental MT in maternal to fetal metal transfer is unclear.

The present study was therefore conducted to determine the role of MT in regulating maternal to fetal transfer Cd and essential micronutrients. As cadmium induces metallothionein synthesis at the transcriptional level, we investigated the relationships between genetic polymorphisms of the region around the transcriptional start site (a known core promoter region) of the metallothionein-2A (MT2A) gene in maternal blood and the levels of metal in maternal blood, umbilical cord blood and placental tissues. To our knowledge, this is the first study to investigate the role of MT2A −5A/G core promoter region single nucleotide polymorphism (SNP) (rs28366003; GeneID, 4502; accession no. NM_005953) in accumulating placental Cd and transferring maternal to fetal essential micronutrients.

MATERIAL AND METHODS

Study Subjects

The study population was included 95 mother–newborn pairs. The women in this study ($n = 95$ with an age range of 18–41 years) consisted of consecutive cases coming to the Gynecology Department of Ankara University's Faculty of Medicine, from September 2009. Inclusion criteria included healthy pregnant women who had been living in Ankara for more than 3 years without a history of smoking, drugs or chronic disease. In addition, there was no history of occupational exposure to cadmium in any of the participants. Infant characteristics such as gestational age, birth weight, birth length and head circumference were recorded. Placenta, maternal and umbilical cord blood were collected at delivery by cesarean section or spontaneous labor. Informed consent was obtained from all participants. The ethics committee of the institution approved the research project (approval no. 152-4828 in 2009) and the questionnaire, which included medical and dietary history as well as data relevant to occupational or possible environmental sources of cadmium exposure.

Sample Collection

A 2 ml aliquot of venous blood was taken from all of the 95 pregnant women before delivery using an EDTA (Vacuette Granier bio-one) during the opening of the routine vascular line. During delivery, 2 ml of venous blood was taken from the umbilical cord into tubes with EDTA and stored at +4 °C. Immediately after delivery, placenta samples were collected and placed in a polyethylene bag. All placenta samples were stored at −20 °C until metal levels were measured.

Determination of −5 A/G SNP in MT2A Gene by the Restriction Fragment Length Polymorphism Method

Genomic DNA was isolated from 100 µl whole blood samples using a Qiagen QIAamp DNA Mini Kit according to the method recommended by the manufacturer. MT2A gene −5 A/G SNP was genotyped using our previous study method (Kayaaltı and Söylemezoğlu, 2010). In order to screen for −5 A/G polymorphism of MT2A, the 241 bp fragment was amplified by polymerase chain reaction (PCR) using the following primers: forward: 5′-CGC CTG GAG CCG CAA GTG AC-3′; and reverse, 5′-TGG GCA TCC CCA GCC TCT TA-3′. The PCR product (241 bp) was then digested with BsgI (New England Biolabs, Hertfordshire, UK) and incubated at 37 °C for 1.5–2 h. The undigested polymerase chain reaction product and digested products were separated on a 2% agarose gel electrophoresis, visualized by ethidium bromide staining under an ultraviolet illuminator, scanned and photographed using the Syngene monitoring system (Fig. 1).

Determination of Cd, Zn, Cu and Fe Levels for Placenta Tissues, Maternal and Umbilical Cord Samples

A microwave system (CEM Mars Xpress) was utilized for digestion of the placentas with concentrated nitric acid solution. The analysis was carried out with a dual atomic absorption system including flame and graphite furnace setups (Varian 240). Each placenta sample was dried for 24 h at 75 °C before reaching a constant weight ranging from 1.0 to 0.8 g. Dried placenta

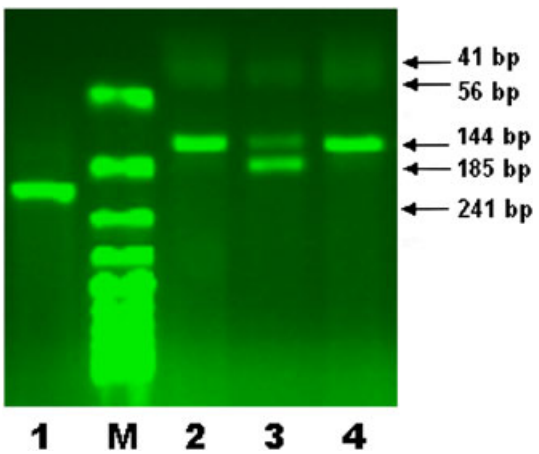

Figure 1. A representative agarose gel image of digested and undigested PCR products with BsgI for MT2A polymorphism: M, 100 bp ladder; lane 1, undigested PCR product; lanes 2 and 4, homozygote typical genotype (AA); lane 3, heterozygote genotype (AG).

samples and blood samples were dissolved in 10 ml of nitric acid and transferred to Teflon tubes, then digested in microwave at 200 °C for 20 min. Digested sample solutions were diluted before being introduced to a graphite furnace. The diluted samples were investigated with flame atomic absorption spectrometry for Zn, Cu and Fe. An atomic absorption spectrophotometer equipped with a graphite furnace and Zeeman background correction system was used for Cd determination. All samples were certificated with Seronorm™ Trace Elements Whole Blood L-2 (reference no. 201605).

Statistical Analyses

Statistical analyses were performed by using the SPSS 16.0 package program. The frequencies of MT2A alleles and genotypes were obtained by direct count, and the departure from the Hardy–Weinberg equilibrium was evaluated by the χ^2 test. The Mann–Whitney *U*-test was used to compare two independent groups and a median (minimum–maximum) was used for descriptive statistics. For determination of the association between two variables, the Spearman correlation coefficient was used, and $P < 0.05$ was considered as statistically significant.

RESULTS

The study was undertaken with 95 healthy mothers who gave informed consent for their 95 newborns. The mean age of these women was 29.7 ± 5.0 years (ranging from 18 to 41) and none of them smoked. A total of 83 pregnant women were identified to possess a homozygote-typical genotype and 12 pregnant women were seen to have a heterozygote for MT2A polymorphism. No homozygote-atypical pregnant women for the MT2A polymorphism participated in the study. The genotype and allele frequencies were consistent with Hardy–Weinberg equilibrium. The Hardy–Weinberg exact test *P*-value was 0.69. Maternal blood Cd levels were statistically higher for mothers with a heterozygote genotype compared with homozygote genotype. In contrast, placental Cd levels were significantly higher in mothers with a homozygote rather than a heterozygote genotype. When placental transfer of Cd was investigated by determining the Cd concentration ratio of placenta to fetus, the ratio was found to be about 20 for homozygote genotype mothers and eight for heterozygote genotype mothers ($P < 0.05$; Table 1). Although the placental Cd level of heterozygote genotype mothers was negatively associated with Zn in umbilical cord blood ($r = -60.9$, $P < 0.05$), none of these metal nutrient/pollutant ratios showed important correlation with birth weight, birth length and head circumference ($P > 0.05$) both in heterozygote and homozygote genotype mothers. No significant difference in umbilical cord blood Cd, Zn and Cu levels between the newborns of homozygote and heterozygote genotype mothers ($P > 0.05$). However, the mean value of umbilical cord Fe levels for newborns with mothers of a heterozygote genotype was higher than others ($P < 0.05$; Table 4). MT2A core promoter region polymorphism and metal concentrations of maternal blood, umbilical cord blood and placenta are presented in Tables 1–4. The certificated value for determination of cadmium levels in the placenta tissues, maternal and umbilical cord blood was found to be 5.7 ± 0.9 ppb, which was within the certificated range (5.1 ± 2.3 ppb).

Table 1. MT2A core promoter region polymorphism and Cd concentrations of maternal bloods, umbilical cord bloods and placentas (in dry placentas)

Genotypes of MT2A polymorphism	*N*	Maternal blood Cd levels (μg dl^{-1})				Umbilical cord blood Cd levels (μg dl^{-1})				Placental tissue Cd levels (μg kg^{-1})			
		Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum
Homozygote typical (AA)	83	1.60 ± 0.94	1.33	0.60	7.42	0.95 ± 0.32	0.88	0.29	1.88	20.83 ± 19.75	13.34	1.67	82.89
Heterozygote (AG)	12	2.54 ± 2.72	1.61	0.71	9.84	0.98 ± 0.28	0.97	0.42	1.53	8.65 ± 6.70	4.77	3.08	22.59
P		<0.05				>0.05				<0.05			

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Table 2. MT2A core promoter region polymorphism and Zn concentrations of maternal bloods, umbilical cord bloods and placentas (in dry placentas)

Genotypes of MT2A polymorphism	*N*	Maternal blood Zn levels (μg dl^{-1})				Umbilical cord blood Zn levels (μg dl^{-1})				Placental tissue Zn levels (μg kg^{-1})			
		Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum
Homozygote typical (AA)	83	4.33 ± 1.13	4.45	1.40	6.75	1.32 ± 0.55	1.25	0.07	3.97	50.46 ± 10.08	50.98	22.98	74.93
Heterozygote (AG)	12	4.82 ± 1.44	4.65	2.19	7.00	1.48 ± 0.53	1.68	0.54	2.11	46.13 ± 7.08	46.72	31.18	59.01
P		>0.05				>0.05				>0.05			

Table 3. MT2A core promoter region polymorphism and Cu concentrations of maternal bloods, umbilical cord bloods and placentas (in dry placentas)

Genotypes of MT2A polymorphism	*N*	Maternal blood Cu levels (μg dl^{-1})				Umbilical cord blood Cu levels (μg dl^{-1})				Placental tissue Cu levels (μg kg^{-1})			
		Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum
Homozygote typical (AA)	83	1.67 ± 0.34	1.61	0.69	2.80	0.69 ± 0.25	0.66	0.03	1.19	5.90 ± 2.59	5.06	2.45	16.23
Heterozygote (AG)	12	1.84 ± 0.50	1.89	1.13	3.01	0.69 ± 0.28	0.74	0.40	1.34	6.63 ± 1.73	7.01	4.13	9.62
P		>0.05				>0.05				>0.05			

Table 4. MT2A core promoter region polymorphism and Fe concentrations of maternal bloods, umbilical cord bloods and placentas (in dry placentas)

Genotypes of MT2A polymorphism	*N*	Maternal blood Fe levels (μg dl^{-1})				Umbilical cord blood Fe levels (μg dl^{-1})				Placental tissue Fe levels (μg kg^{-1})			
		Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum	Mean ± SD	Median	Minimum	Maximum
Homozygote typical (AA)	83	343.21 ± 89.50	353.68	76.80	518.50	270.90 ± 129.81	251.06	59.53	595.09	526.78 ± 194.32	478.42	94.94	1414.55
Heterozygote (AG)	12	373.36 ± 102.87	388.63	181.84	593.98	455.89 ± 214.17	438.76	93.39	781.94	623.50 ± 161.96	576.03	458.71	1023.95
P		>0.05				<0.05				>0.05			

DISCUSSION

In the present study, cadmium concentration in the umbilical cord blood was low in both genotypes compared with concentrations in maternal blood and placenta, which indicates that only a small amount of Cd was transferred to the fetus. Additionally, there was no significant difference in umbilical cord Cd levels between the newborns of mothers with an MT-homozygote genotype and those of the MT-heterozygote genotype. Maternal blood Cd levels were statistically higher for mothers with a heterozygote genotype than those of mothers with a homozygote genotype for MT2A polymorphism while placental Cd levels were significantly higher in mothers with a homozygous compared with the heterozygous genotype. The ratio of placental cadmium to umbilical cord Cd concentration was clearly higher in mothers having a homozygote genotype than others. One explanation may be that the cadmium in the blood of MT-heterozygote genotype women could not be retained because of the relative lack of MT in the placenta due to the presence of the −5A/G SNP in the human MT2A gene, which can limit MT expression compared with MT levels in the same organ of an MT-homozygote genotype. These findings suggest that endogenous placental MT may play an important role in preventing maternal-to-fetal transfer of cadmium. A notable finding was the decrease in Zn concentration observed in umbilical cord blood with increasing placenta Cd for newborns of mothers with a MT-heterozygote genotype but not with a MT-homozygote genotype. Although the specific mechanism behind an interaction of Cd and Zn in the placenta is not completely understood, several studies have suggested an involvement of MT. The accumulation of Cd in placenta induces the synthesis of the metal binding protein metallothionein, which may be responsible for binding more zinc, thereby retaining this element inside the placental cells. Under these circumstances, an inadequate amount of zinc might be transferred to the fetus, which is likely to affect fetal growth and development (Kuhnert *et al.*, 1987; Ronco *et al.*, 2006). In our study, none of the observed metal nutrient/pollutant occurrences showed important correlation with birth weight in both heterozygote and homozygote genotype mothers. However, low placental nutrient/pollutant ratios could be considered as an additional detrimental factor affecting fetal growth for newborns of mothers with an MT-homozygote genotype. The major functions of MT are known to be the maintenance of Zn homeostasis and protection of cells from the toxicity of metal, including Cd. Also, transgenic mice that constantly overexpress metallothionein genes have been found to be cadmium tolerant. In contrast, knockout mice with defective metallothionein genes are more sensitive to cadmium toxicity than wild-type mice (Palmiter *et al.*, 1993). In humans, Kayaaltı *et al.* has found that cadmium levels in autopsy kidney increased significantly with the core promoter region polymorphism of metallothionein 2A (Kayaaltı *et al.*, 2010). It is therefore believed that MT deficiency probably enhances Cd sensitivity. In addition, the −5 A/G SNP in the human MT2A gene can limit MT expression (Kita *et al.*, 2006). Low MT expression would theoretically predispose people to Cd toxicity, as appears to be the case for arsenic toxicity and low MT. As cadmium induces metallothionein synthesis at the transcriptional level, we analyzed genetic polymorphisms of the region around the transcriptional start site (a known promoter region) of the metallothionein-2A gene. Additionally, the MT2A gene is the main isoform of human metallothionein genes and the expression of MT2A constitutes 50% of the total expressions of all metallothionein isoforms (Skroch *et al.*, 1993). Therefore, in the present study, the −5A/G core promoter region was chosen for the investigation of SNP in MT2A (rs28366003) due to almost complete loss of functionality in metal toxicokinetics and thus might exert a stronger biological effect. We also observed that the rate of Cd accumulation significantly changed due to maternal genetic background and a consistent association with a higher Cd retained by MT in the placenta was seen in mothers with a MT-homozygote genotype. However, we did not observe any association between maternal genetic background and Zn or between Cd and Zn in the placenta. Despite the fact that cadmium is a much stronger inducer of MT than Zn and has a higher affinity for MT (Klaassen *et al.*, 1999), our findings provide no evidence for an involvement of Cd-induced MT in the impaired transfer of Zn to the fetus. Thus, there seems to be other mechanisms involved in the Cd-Zn interactions aside from MT. This result is in agreement with another study (Kippler *et al.*, 2010). Also, the Cd-induced reduction ZnT-1 gene expression in mice has been seen to result in reduced levels of Zn available for the conceptus (Fernandez *et al.*, 2007). In this study, it has been stated that the side effects of Cd accumulation or altering micronutrient levels on the newborns are not apparent at the time of delivery, but subclinical toxicity might be possible. This may be due to the differences in Cd exposure route and the relatively low Cd level of exposure compared with those observed in other studies. In our study, the median Cd concentration in the placenta (20 μg kg^{-1} dry weight) was similar in the placenta of nonsmoking women in nonpolluted areas, where placental Cd concentrations are reported to be around 20–40 μg kg^{-1} dry weight or around 5 μg kg^{-1} wet weight (Iyengar and Rapp, 2001; Korpela *et al.*, 1986; Kuhnert *et al.*, 1987; Odland *et al.*, 2001, 2003, 2004; Osman *et al.*, 2000). Owing to the low placental Cd level observed in our study, further studies involving women with higher Cd levels are needed to quantify the adverse effects of Cd exposure during pregnancy. Novel information obtained in the present study was the higher umbilical cord Fe levels observed in newborns of mothers having an MT-heterozygote genotype. An overload of Fe in umbilical cord blood may be associated with toxicity. In addition and due to the opposite behavior of Zn and Fe, increasing Fe concentration in the placenta of mothers of a heterozygote genotype can decrease the ratio of zinc in umbilical cord blood.

In conclusion, Cd exposure even at environmental levels may be associated with deleterious effects as a result of altering umbilical cord micronutrient levels for newborns with mothers of a heterozygote genotype. Further investigation is necessary to better understand the reduced flux of maternal Zn and increased flux of maternal Fe to the embriyonal compartment observed as a result of Cd exposure in newborns of mothers with a heterozygote genotype.

Conflict of interests

The authors declare that they have no conflict of interest.

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