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Microarray analysis of metallothioneins in human diseases– A Review

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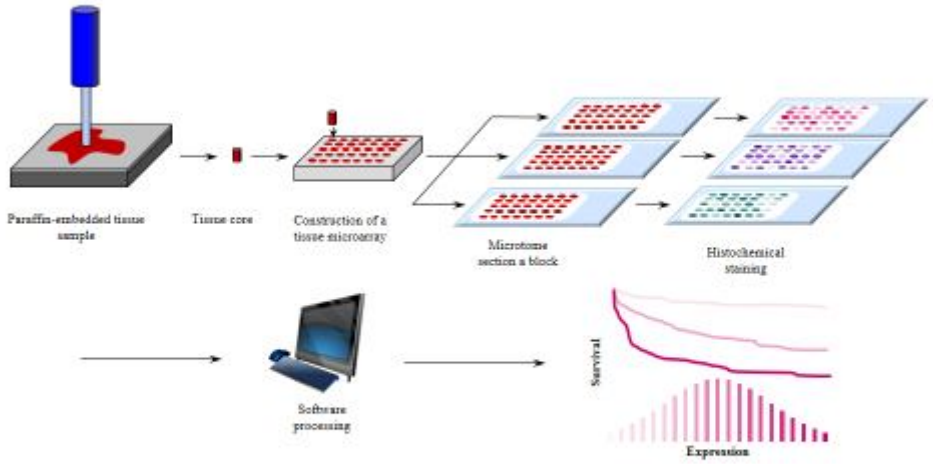
Graphical abstract



Highlights

- We summarized microarray for metallothionein detection.
- The fields of applications are mentioned.
- The clinical biomarkers possibilities are discussed.

Abstract

Metallothioneins (MTs), low molecular mass cysteine-rich proteins, which are able to bind up to 20 monovalent and up to 7 divalent heavy metal ions are widely studied due to their functions in detoxification of metals, scavenging free radicals and cells protection against the oxidative stress. It was found that the loss of the protective effects of MT leads to an escalation of pathogenic processes and carcinogenesis.

The most extensive area is MTs expression for oncological applications, where the information about gene patterns is helpful for the identification biological function, resistance to drugs and creating the correct chemotherapy. In other medical applications the effect of oxidative stress to cell lines exposed to heavy metals and hydrogen peroxide is studied as well as influence of drugs and cytokines on MTs expression and MTs expression in the adipose tissue. The precise detection of low metallothionein concentrations and its isoforms is necessary to understand the connection between quantity and isoforms of MTs to size, localization and type of cancer. This information is necessary for well-timed therapy and increase the chance to survival. Microarray chips appear as good possibility for finding all information about expression of MTs genes and isoforms not only in cancer, but also in other diseases, especially diabetes, obesity, cardiovascular diseases, ageing, osteoporosis, psychiatric disorders and as the effects of toxic drugs and pollutants, which is discussed in this review.

List of abbreviations: HCC – hepatocellular carcinoma, HFD – high fat diet, MMP - matrix metalloproteinase, MT – metallothionein, MTF – metal transcription factor, MRE – metal responsive element, ROS – reactive oxygen species.

Keywords: drug; gene expression; metallothionein; microarray; resistance; tumour diseases

1. Introduction

1.1. Metallothioneins

Metallothioneins (MTs) are a group of low molecular mass cysteine-rich proteins, which were isolated from horse kidney by Margoshes and Vallee in 1957 [1]. These proteins are able to bind up to 20 monovalent and up to 7 divalent heavy metal ions [2] and their main functions include metal ions detoxification of the organism [3] and scavenging free radicals to protect cells against oxidative stress [4]. It was found that the loss of the protective effects of MT leads to an escalation of pathogenic process (Fig. 1). Moreover, there is increasing number of papers aimed at describing the role of these proteins in anticancer therapy [5-10].

1.2. Isoforms of metallothioneins

Generally, mammalian metallothioneins isoforms could be divided into four groups as MT-1, MT-2, MT-3 and MT-4 [11]. The expression and localization of individual MT isoforms vary at intracellular level (cytosol, nucleus, lysosomes, and mitochondria) and in individual tissues. In human, eight members of MT-1 (MT-1A, 1B, 1E, 1F, 1G, 1H, 1M, 1X) one member of MT-2A [12,13], MT-3 and MT-4 [14] have been discovered. These isoforms have differing rates of degradation and could be distributed in various ratios in individual tissues. The general physicochemical properties of MT isoforms are similar, however there is some specialisation of biological function [15]. The most widely expressed isoforms in the body are MT-1 and -2. These occur in tissues of kidney, liver, intestine and pancreas. MT-1A, E, X and MT-2A isoforms were found in normal prostate tissue [3]. MT-2A isoform is also connected

with the proliferative activity of human prostate cancer cells [16] and breast cancer [17]. Besides, metallothionein 2A's polymorphs were studied for their association with the risk for a variety of diseases such as prostate cancer, atherosclerosis, stroke and diabetes [3,18]. MT-3 is found mainly in the brain, but it is expressed also in heart, kidney, and stomach in trace amounts [19]. MT-4 can be detected in epithelia and the maternal deciduae [20].

1.3. Metallothionein expression

Metallothionein expression is induced by numerous factors such as stress hormones, cytokines, reactive oxygen species (ROS), radioactive and UV irradiation and exposition to metal ions [21]. The metallothionein expression follows after the binding of transcription factor MTF-1 (metal regulatory-transcription factor-1) to the regulatory part MRE (metal responsive element), which is localized at promotor of MT-1 gene. MTF-1 contains six Zn-fingers and in the cell it is stored in inactive form coupled with its inhibitor MTI [22]. Metal ions come into the intracellular space, where they are bound to MTI and by this way MTF-1 is activated and is able to induct expression of MTs by the attaching to MRE [23]. After the transcription and translation the posttranslational modifications such as phosphorylation, glycosylation, deamination and oxidation become crucial [24]. Other heavy metals are also able to induce MTs transcription via MRE, however only Zn and Cd are able to activate MTF-1 [25].

Higher level of MT was discovered in proliferating cells. This fact should be caused by the increased need for Zn and DNA protection against ROS species [26]. The study of

metallothionein expression at mRNA level shows the role of this protein in the cell protection to high zinc concentration [27]. MT can also control the activity of zinc fingers by competition about Zn. It was shown that regulating the availability of zinc the thionein/Zn-MT conjugate pair modulates the DNA-binding activity of zinc finger transcription factors [28]. The feasibility of exchanging of Cd^{2+} bound to transcription factors to Zn^{2+} by MT thereby restoring the DNA-binding activity of transcription factors is an important event of metal detoxification [28]. Under physiological conditions, the role of metallothionein is zinc reservoir for zinc-dependent proteins.

At the protein level, MT has been examined as a marker of cancer [29] and also of metals' and toxic compounds' intoxication [30,31]. For these purposes, numerous analytical and biochemical methods are used [32,33], whereas the electrochemical ones seem to be the most sensitive [33-35]. Nevertheless, the tissue distribution can be well detected by immunohistochemistry, which was based for performing meta-analysis of metallothionein as an immunohistochemical marker of cancer [36]. Based on the published data it can be concluded that MT could be considered as a marker of tumour diseases, however, combination of mRNA and protein levels is needed [37,38]. Therefore, we aimed our attention at summarization of MT mRNA determination.

2. PCR and RT-PCR

Genotyping of various MT forms can be performed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) technique. Using this method Krześlak et

al. investigated three known single nucleotide polymorphisms (SNP) of MT2A gene in patients with breast cancer [39]. They found that the rs28366003 SNP of MT2A is positively associated with breast cancer. In an in vitro study it could be shown that MT-2A induces MMP-9 up-regulation through the activation of NFκB and Ap-1 signalling pathways, which can facilitate the migration and invasion of breast cancer cells [17]. Earlier, the same SNP had been shown to be linked with higher MT2A mRNA expression in prostate cancer tissue [3]. Gene expression is obviously analysed by quantitative (real-time) reverse transcription polymerase chain reaction (qRT-PCR). Comparative analysis of transcriptomes of non-malignant bone and osteosarcoma revealed that members from metallothionein groups such as MT-1E, MT-1H, MT-1X, MT-2A, MT-1B, MT-1G and MT-1L were up-regulated in osteosarcoma samples [40]. Three isoforms, MT-1E, MT-1H and MT-1X were among the ten most highly up-regulated genes between malignant tissue and non-malignant bone. RT-PCR was used to validate the differential expression of MT-1E [40]. Szelachowska et al. studied the mRNA levels of MT-1F, MT-1X and MT-2A like an evaluation of the radiotherapy effects on expression of metallothionein isoforms in human rectal adenocarcinoma [41]. They found high expression of MT-1F followed by MT-1X and MT-2A in carcinoma cells. All studied isoforms had even higher expressions after radiotherapy, but the change was not statistically significant [41]. MT protein expression (MT1/2) was also evaluated. There was no correlation between mRNA and protein levels [41]. Fu et al. studied MT-1G expression in primary thyroid cancers by qRT-PCR [42]. Comparing malignant and non-malignant thyroid tissues the down-regulation of MT-1G could be detected in cancer cells. Cell cycle arrest and

apoptotic cell death were induced and the migration and invasion were inhibited in thyroid cancer cell lines transfected with MT1G supporting the tumour suppressor function of this gene [42]. Based on these studies, it clearly follows that the expression of MT isoforms is interesting; however, a clear picture of more than one isoform is crucial. For studying more isoforms simultaneously, microarray technology can be useful. Therefore, the summary of this method for MT isoforms detection follows.

3. Microarray

3.1. Introduction to microarray technology

Microarray is a relatively new technology for detection and characterization of DNA. This high-throughput genome analysis can be applied for studying DNA-protein interaction, transcription analysis, detection and characterization of genetic variants [43] and environmental toxicology [44]. The summary of microarray analysis is shown in Fig. 2. The basic description of DNA microarray is a collection of microscopic DNA spots attached to a solid surface [43]. One spot contains picomole amount of a specific DNA probe. Probes can be a short section of a gene or other DNA element used for hybridization of cDNA or cRNA in the sample (target) under high-stringent conditions. The probes are synthesized and then attached via surface engineering to a solid surface by a covalent bond to a chemical matrix in common microarrays. The solid surface can be glass or a silicon chip, in which case they are colloquially known as an Affy chip when an Affymetrix chip is used. Other microarray platforms use microscopic beads, instead of the large solid support (such as Illumina).

Alternatively, microarrays can be constructed by the direct synthesis of oligonucleotide probes on solid surfaces. DNA arrays are different from other types of microarrays only in that they either measure DNA or use DNA as part of its detection system (http://www.gene-chips.com/). Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labelled targets to determine relative abundance of nucleic acid sequences in the sample. Relatively new is the electrochemical detection of probe-target hybridization based on enzymatic labelling of the target sequence. Numerous commercial microarray platforms covering human or model animal genomes are commercially available.

Tissue microarrays are based on multiple immunohistochemical detection of proteins in up to 1000 tissue cores in diameter of 0.6 mm (Fig. 3). These tissue cores are inserted into a paraffin block in an array pattern. Sections from this block are cut using a microtome, placed on a microscopic slide and analysed by any method of histological analysis. Other variation is frozen tissue microarray, where the frozen tissue cores in diameter of 2 mm are embedded in an optimal cutting temperature block [45].

The use of microarrays has several advantages over traditional methods of proteins and nucleic acids detection. Large number of the samples can be simultaneously analysed with commercially available genomic slides.

Microarrays allow us to monitor global changes in gene and protein expression. The signal is detected, quantified, integrated and normalized with software and reflect the gene expression profile or molecular portrait of given biological sample. However, this molecular portrait is

determined by the number and type of probes spotted on a slide, thus in traditional microarrays, only molecules that are searched, can be found [46].

Microarray studies are still limited by access and cost, especially for non-mainstream samples. Tissue samples usually represent a mixture of different cell types, thus the observed global changes in expression profiles reflect the entire cell types present in sample. In some types of sample, especially early stages of tumours there is often problem with low amount of tissue available. Nucleic acids and protein microarrays are destructive methods – the cells are disrupted and further processed. In DNA and RNA microarrays the quality of nucleic acids and amplification steps may lead to distortion of expression profiles. In protein microarrays there are no amplification methods available, thus a high requirement on detection step is taken and low-abundant proteins may be misrepresented [47].

The described techniques have been widely used in many fields allowing quick analysis of multiple samples studying the expression of thousands genes in a small sample volume. Thereby, we are describing highly throughput type of analysis. For this reason microarrays are abundantly used for comparative genomes hybridization and expression profiling in different types of samples. This results in finding of candidate genes, whose expression needs to be confirmed by other methods, such as qPCR, Western blot or RNA silencing (Fig. 4).

3.2. Microarray-based analysis for detection of metallothioneins

The aim of this review is to summarize articles on using microarray for studying MT expression within the period from 2010 to 2014. According to our best knowledge, there have

been published 46 papers covering the topic of this review (Table 1). These were separated into oncological application, and other medical application.

3.2.1. Oncological application

The detection of metallothionein by microarray is spread in oncological studies. The information about gene patterns is helpful for the identification biological function, resistance to drugs and creating the correct chemotherapy protocol. The most studied are tumours of head and neck, prostate carcinoma, ovarian carcinoma, hepatic tumours and colorectal carcinoma. Except human samples and cell lines the animal cancer models and also the action of carcinogens and side effects of cytostatics have been explored.

3.2.1.1 Head and neck tumours

In head and neck tumours metallothioneins were studied in connection with resistance to chemotherapeutics. In tongue squamous cell carcinoma the mechanisms of drug resistance were studied using sensitive (Tca8113) and pingyangmycin-induced multidrug-resistant (Tca8113/PYM) cell lines [48]. The resistance to cisplatin, pirarubicin, paclitaxel, adriamycin and mitomycin was investigated. By cDNA microarray it was found that MT-2A, MT-1B and MT-1K are up-regulated in Tca8113/PYM and it seems that the resistance of the cell line could be related with MT-2A. In further microarray analysis using the same cell lines MT-1X has been shown as a mediator of a novel gene TCRP1 (tongue cancer resistance-associated protein) associated resistance to cisplatin [49].

3.2.1.2 Prostate and ovarian cancer

Copper homeostasis disturbances are involved in development of prostate disorders. Bigagli et al. investigating the 24 h influence of Cu within the very low concentration range from 10^{-17} to 10^{-6} M to a non-neoplastic adult human epithelial prostatic cell line RWPE-1 showed that transcriptional regulation of MT has an important role in the tight control of intracellular free Cu (II) levels [50]. Microarray data demonstrated concentration dependent changes in the expression of various MT isoforms. MT-1B, MT-1E, MT-1G and MT-1H were down-regulated in the lowest concentrations of Cu and up-regulated between 10^{-14} and 10^{-6} M. MT1-M and MTF1 also was down-regulated at lower concentrations and up-regulated within the concentration range from 10^{-9} to 10^{-6} M. MT-1A and MT-2A were up-regulated at all concentration in Cu-treated cells compared to cells without treatment (control).

Using a dog animal model transcriptome analysis of sequential biopsies representing the onset of benign prostatic hyperplasia has been performed to characterize the gene expression pattern associated with prostatic hyperplasia [51]. A number of genes involved in detoxification, cell movement, calcification, matrix remodelling, mucosa protection, transdifferentiation, senescence and apoptosis programs have been identified as altered, including MT-2A. Han et al. (2013) analysed MT-1H expression in 30 sets of microarray data of human malignancies [52]. A consistent down-regulation of MT-1H seems to be present in various types of malignant tumours compared with normal tissues. For further validation MT-1H *in situ* hybridization was performed on tissue microarray containing normal prostate tissues and

prostate cancer samples [52]. Significantly decreased MT-1H expression has been found in cancer tissues compared with normal prostate, and low level of expression in tumours was associated with poor clinical outcome. *In vitro* MT-1H has been shown to exert tumour suppressor effect on prostate cells via interacting with euchromatin histone methyltransferase 1.

In tissue microarray analysis of primary ovarian cancer samples the negative nuclear expression of metallothionein protein has been demonstrated to predict a better therapeutic response to adjuvant platinum-based chemotherapy and improved progression-free survival of patients [53].

3.2.1.3 Hepatic tumours

Genes that could be used as markers of liver fluke-associated intrahepatic cholangiocarcinoma, were analysed by Subrungruang et al. [54]. More than 3 000 genes were found to be up- or down-regulated in cholangiocarcinoma tissues compared to normal liver tissue samples. Genes from metallothionein family (namely MT-1A, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X and pseudogene MT1-IP) were down-regulated and showed significant changes in expression.

Total RNA and genomic DNA were extracted from hepatocellular carcinoma (HCC) and non-malignant liver tissue for gene expression profiling and SNP chip array analysis by Kanda et al. [55]. Double array analysis method with complementary qRT-PCR and methylation-specific PCR (MSP) was suitable for identification of metallothionein 1G as tumour

suppressor gene and to show that it is silenced in HCC by promoter hypermethylation [55]. Later on, the expression of MT1/2 was evaluated immunohistochemically in tissue microarrays containing samples from HCCs, adjacent noncancerous livers, and normal livers [56]. This study demonstrated the loss of nuclear and cytoplasmic expression of MT1/2 in HCC compared with adjacent noncancerous liver tissues and that the loss of nuclear MT1/2 expression is an independent prognostic indicator of poor recurrence-free survival and overall survival in patients with HCC [56].

Furthermore, the toxicity of cadmium was studied on human hepatoblastoma cells (HepG2) by the Agilent Whole Human Genome Oligo Microarray [57]. The cells were exposed to 2 and 10 µM concentration of $CdCl_2$ for 24 h. At the higher concentration a number of genes related to cancer development were up-regulated and many genes connected to liver function were down-regulated, whereas at the lower concentration the up-regulation of various metallothionein isoforms (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1J (pseudogene), MT-1M, MT-1L (gene/pseudogene), MT-1X, and MT-2A) was demonstrated as a protection mechanism against cadmium toxicity [57].

3.2.1.4 Colon cancer

Microarray analysis of colon cancer samples and adjacent normal mucosa tissues from 40 cancer patients, who had undergone tumour resection without receiving preoperative therapy, was performed [58]. Compared to normal tissues, the gene expression of MT-1F, MT-1G, MT-1X and MT-2A was down-regulated in colon cancer, which could be confirmed by qRT-

PCR [58]. The tissue microarray analysis of samples demonstrated that MT1/2 protein expression was decreased in tissues with down-regulated MT mRNA expression. The results on protein expression were further confirmed by Western blot analysis [58]. Additional analysis of the loss of heterozygosity (LOH) and MSP showed that MT-1F is down-regulated mainly by LOH in colon cancer [58].

The influence of 50 μM rosiglitazone (PPARγ ligand) and 0.1 μM AS601245 (a selective JNK inhibitor) to human colon cancer cells (CaCo-2, HT29 and SW480) after 24 h treatment was studied [59]. It was found that rosiglitazone and AS601245 decrease cell adhesion and migration through modulation of gene expression. Because of similar effect of the treatment on all cell lines the microarray analysis was performed on CaCo-2 cell line only. After treatment with rosiglitazone the increased expression of genes coding for metallothioneins (MT-1X, MT-1E, MT-1G, MT-1H, MT-2A, MT-1M) was observed compared to untreated cell line. After combination with AS601245 metallothionein genes were still induced compared to untreated cells, but to a lesser extent than after treatment with rosiglitazone only [59].

3.2.1.5 Other solid tumours

Statistical analysis of the osteosarcoma transcriptomes from biopsy samples found differential expression of several metallothionein family members [40]. MT-1E, MT-1H, MT-1X, MT-2A, MT-1B, MT-1G, and MT-1L, were up-regulated in osteosarcoma, and three (MT-1E, MT-1H and MT1-X) were among the 10 most highly up-regulated genes. Noteworthy, there

was no correlation between MT expression and chemoresistance in osteosarcoma [40]. In another study the gene expression of 73 high-grade soft tissue sarcoma samples was analysed by cDNA Microarray and clustered by complete-linkage hierarchical clustering [60]. The ratio of the gene expression in the sample relative to the average signal of expression of all genes examined was determined. Importantly, the samples of patients with the highest rate of metastases were characterized by MT-2A, MT-1X, MT-1F and MT-1H over-expression [60]. Scaruffi et al. investigated the transcriptome of resident bone marrow cells from localized and metastatic neuroblastoma patients compared to healthy subjects [61]. They found an altered gene expression in bone marrow cells including over-expression of metallothioneins.

The biology behind ependymoma recurrence was also studied by expression microarray [62]. The expression of metallothioneins (MT-1L, MT-1G, MT-1E, MT-1X, MT-1B, MT-2A, and MT-3) was down-regulated in 65 to 85 % of relapses depending on the MT compared with initial tumours thereby representing a group of the most homogenously differentially expressed genes in recurrences. The neural growth inhibitory factor MT-3 was the most frequently down-regulated gene among metallothioneins, which was confirmed by qRT-PCR and immunohistochemistry [62]. In addition, tissue microarray analysis in an independent cohort of paediatric ependymomas demonstrated lower MT-3 protein expression at relapse compared to diagnosis in 70.8% of patients [62].

Using tissue microarray it was found that over-expression of MT1/2 is significantly more frequent in primary cutaneous malignant melanoma with haematogenous metastases [63].

3.2.1.6 Haematological malignancies

Gene expression profile of human myeloid leukaemia cells K562, namely PU.1-knockdown K562 cells versus control and PU.1-overexpressing K562 cells versus control, was investigated by microarray analysis [64]. The interest was focused on PU.1-transcription factor for haematopoiesis, since down-regulation of PU.1 seems to be related to development of various haematological malignancies. It was found that MT-1G and MT-1A were distinctly induced in PU.1-knockdown K562 cells [64]. Furthermore, negative correlations of PU.1 expression with the MT-1G and MT-1A expressions, respectively, could be confirmed by analysing leukemic bone marrow cells derived from AML patients [64].

3.2.1.7 Carcinogens and anticancer drugs

Changes in the human genome-wide transcriptome of H9 human embryonic stem cell (hESC) line due to the exposure to 1 Gy of gamma-radiation were detected at 2 and 16 h post-irradiation [65]. This study showed the over-expression of many genes including metallothioneins (MT-1M, MT-1L, MT-1H and MT-1G) in irradiated hESC at 16 h post-irradiation.

The induction of MT isoforms in response to anticancer drugs e.g., doxorubicin has been reported [66]. Microarray analysis can also be used to identify the target genes of MT. The influence of MT against cardiotoxicity of doxorubicin was studied on wild type (MT+/+) mice in comparison with MT-1/2 null (MT-/-) mice [66]. Global gene expression profiles of cardiac cells from two genotype mice have been analysed on the 4^{th} day after doxorubicin (15 mg/kg,

i.p.) or equal volume of saline administration. 381 characteristically MT-responsive genes such as *map3k6*, *fos*, *ucp3*, *car3*, and *atf3* were identified in response to doxorubicin [66].

3.2.2. Other medical application

Expression of metallothioneins is generally associated with oxidative stress in obesity, cardiovascular diseases, diabetes, osteoporosis, vision, but also with hepatotoxic and nephrotoxic effects of heavy metals and drugs. The role of metallothioneins in ageing, and psychiatric disorders is studied, too.

3.2.2.1 Obesity

Metallothioneins are expressed and upregulated e.g., by glucocorticoids in human adipose tissue [67]. MT-1 and MT-2 were also identified as hepatic glucocorticoid-regulated target genes using whole genome gene expression microarray [68]. Interestingly, MT-1 and -2 knockout mice are moderately obese suggesting the role of these proteins in the regulation of energy balance [67]. Cui et al. investigating spleen oxidative stress induced by high-fat diet (HFD) showed the down-regulation of MT-1 gene in the HFD (21.2% fat, w/w) mice compared with the controls (4.9% fat) [69]. In another study, male C57BL/6 mice were exposed to dietary restriction (75% of normal diet for 6 months) followed by 6 months of *ad libitum* refeeding. This group was compared with continuously *ad libitum* fed control group [70]. Dietary restriction resulted in lower body weight. Transcriptome analysis in mouse liver was done and 239 and 184 genes were at least two times up- or down-regulated in dietary

restriction compared to *ad libitum* fed control group [70]. MT-1 was increased in the group with dietary restriction.

3.2.2.2 Cardiovascular diseases

In the adult the protective and antioxidative effect of high density lipoprotein (HDL) on the vascular endothelium is assumed [71]. However, in a microarray study, investigating the effect of foetal HDL, which represents the main lipoprotein class in cord blood and is characterized by high proportion of apolipoprotein E, on human placental endothelial cells (HPEC) showed that foetal HDL reduced the MT1-X and MT-2A expression [71]. Foetal HDL is thought to exert key functions for development and metabolism of foetal tissues, and seems to be particularly important for the development of the central nervous system [71]. The effects of the apolipoprotein A1-rich adult HDL on MT synthesis might be different and still has to be determined.

Cadmium is known to induce vascular diseases such as atherosclerosis. Toxicity of non-lethal dose of cadmium on human coronary artery endothelial cells (HCAEC) was studied by microarray utilizing of OpArrayTM Human V4.0 slide [72]. There were only 3 genes for which the levels of expression increased more than 2-fold, the MT-1E, MT-1H, and MT-1B, which underlines the importance of these MT isoforms in the vascular protection against heavy metal toxicity.

Hyperbaric oxygen treatment (HBOT) seems to have beneficial effects on wound healing and reparative angiogenesis [73]. Global gene expression analysis on human microvascular

endothelial cells exposed to hyperbaric oxygen under conditions similar to a clinical treatment showed that some metallothioneins especially MT-1E, MT-1F, MT-1G, MT-1H, MT-1M and MT-1X were up-regulated immediately after HBOT, some metallothioneins as MT-1E, MT-1F, MT-1H and MT-1X after 24 h [73].

3.2.2.3 Diabetes

Pancreatic beta-cell dysfunction has a central role in the development and progression of type 2 diabetes. Microarray analysis on beta-cells obtained from patients with diabetes of type 2 revealed the significant up-regulation of MT-1E, MT-1M, MT-1X, MT-2A, and the pseudogene MT-1P2 [74]. This finding was assumed to reflect alterations in oxidative stress [74]. Nutrigenomics means the utilization of microarray technique to study the effects of nutrients and food compounds on gene expression thereby better understanding how the nutrients regulate biological processes [75]. Taurine (2-aminoethanesulfonic acid) seems to have a protecting effect against insulin resistance and diabetes mellitus [75]. Microarray analysis on human colon carcinoma derived Caco-2 cells showed the up-regulation of thioredoxin interacting protein (TXNIP) and MT-1H. Therefore, it was concluded that anti-inflammatory and anti-oxidative effects of taurine may partly due to the increased MT-1H expression [75].

In the Nagaya-Shibata-Yasuda (NSY) mouse, which is an animal model of type 2 diabetes, high-sucrose diet increases the glucose intolerance, body weight gain, and induces liver steatosis [76]. Microarray analysis to detect hepatic gene expression levels revealed

significantly lower MT-1 and MT-2 expression in NSY mice compared with control C3H mice, irrespective of diet [76]. Considering the biological modulating activity of MT on liver mitochondria, down-regulation of MT was thought to contribute to the development of steatohepatitis and obesity [76]. Transcriptome analysis on liver has also been determined in C57BL/6J mice fed with a high-glucose diet for 4 weeks [77]. Elevated fatty acid accumulation in liver, insulin resistance at higher body weight have been observed with upregulation of 197 genes and down-regulation of 189 genes in liver [77]. The expression levels of MT-2 decreased compared to control.

3.2.2.4 Hepatotoxicity

Antioxidant and hypolipidaemic activities of a low concentration (300 μg/ml) of *Tamarindus indica L.* fruit pulp methanol extract was tested on human hepatoma HepG2 cell line [78]. Several genes that are related to reactive oxygen species and lipid metabolism showed changes in their expression. MT-1M, MT-1F and MT-1X were up-regulated, but not other MT isoforms [78].

Toxic effects of polychlorobiphenyls (PBC), namely coplanar PCB-77 and non-coplanar PCB-153 on HepG2 cells were studied by microarray analysis [79]. It was shown that both agents induce oxidative stress, but through involvement of different gene sets. PCB-77 triggers the receptor mediated nuclear apoptotic pathway, whereas PCB-153 triggers the mitochondrial apoptotic pathway [79]. Interestingly, MT-1K, MT-1X and MT-1F were involved in the response to PCB-153, but not to PCB-77.

MT-1 and MT-2 are important protective factors against Cd toxicity, however, MT-3 null mice are resistant to Cd hepatotoxicity [80]. Microarray analysis on liver of MT-3 null and wild type mice at 4 hs after 20 μmol/kg Cd injection showed that only 37 genes were differentially expressed [80]. Cd induced up-regulation of the inflammation-associated cytokines serum amyloid A1 and A2 was inhibited in the liver of MT-3 null mice. There has not been altered expression of other MT isoforms [80].

3.2.2.5 Nephrotoxicity

Using cDNA microarray to analyse global gene expressions of cortical renal tissue of mice immunized with cationic-bovine serum albumin to induce membranous glomerulonephropathy (MN) Wu et al. (2011) revealed 175 genes with significantly different expressions compared with normal kidneys [81]. They proposed that some of the differentially expressed genes might be used as biomarkers for human MN and should be tested on human samples. Significant up-regulation of MT-1 in association with MN was confirmed by qRT-PCR [81].

3.2.2.6 Psychiatric disorders

Shelton et al. investigated post-mortem brain tissue samples from 14 depressed persons who were psychotropic drug free at the time of death and age- and sex-matched normal controls [82]. Microarray analysis of Brodmann Area 10 tissue samples using the Affymetrix Exon 1.0 ST arrays showed differential expression of genes that are related to inflammation, apoptosis

and oxidative stress. MT-1M expression was reduced in samples derived from patients with major depression as confirmed by qRT-PCR, which suggests an important role of this isoform in protection against local inflammation and development of depression [82].

Gene expression changes associated with suicide in brains of mood disorder patients were studied by microarrays (Affymetrix HG-U133 Plus2.0) in the dorsolateral prefrontal cortex, in the anterior cingulate cortex and in nucleus accumbens [83]. Several MT-1 and MT-2 isoforms (MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, and MT-2A) were down-regulated in the anterior cingulate cortex of suicide subjects compared with non-suicides, and three of them (MT-1F, MT-1G, and MT-1H) were also down-regulated in the nucleus accumbens. MT-1M and MT-3 showed no differential expression between suicides and non-suicides [83]. It was concluded that failed neuroprotection against stress due to decreased expression levels of MT-1 and MT-2 might be molecular risk factor for suicide [83].

A mouse model consisting of two inbred mouse lines showing high (HAB) and low (LAB) anxiety-related behaviour can be used to analyze the traits of anxiety and depression [84]. In this model Czibere et al. investigated the gene expression in emotion-regulating parts of the limbic system and the brain regions closely connected to them using microarray [84]. More than 300 genes were differentially expressed between HAB and LAB mice in all analysed brain regions. Significantly lower MT-1 expression in HAB mice was confirmed by qRT-PCR [84].

3.2.2.7 Ageing

The protein levels of MT1/2 were studied in ageing skin by tissue microarray [85]. The expression of MT1/2 decreased significantly with increasing age, just like the expression of the proliferation markers Ki-67 and PCNA. The decline was more pronounced in sun-exposed skin [85].

3.2.2.8 Osteoporosis

Genome-wide gene expression was analysed in primary cultures of osteoblasts derived from osteoporotic and non-osteoporotic human bone tissue samples [86]. The microarray data revealed that oxidative stress might be involved in the pathogenesis of osteoporosis. In further *in vitro* experiments the expression of MT-1G was confirmed to be important in response to oxidative stress in bone cells [86].

3.2.2.9 Vision

Whole-genome expression profiling of cornea, retinal pigment epithelium, trabecular meshwork, iris, lens, ciliary body, retina and sclera obtained from human cadaver eyes was examined focusing on MT expression [87]. MT-1A, MT-2A and MT-1X were highly expressed in all the tissues, but in particular in cornea, lens and iris. It might reflect that cornea and lens are natural barriers to external environmental insults e.g., UV radiation. MT-1G was also highly expressed in lens. The expression levels of MT-1E, MT-1F, MT-1M and MT-1G were lower compared to MT-1A, MT-2A and MT-1X [87]. MT-1H and MT-3

isoforms were expressed at very low levels, whereas MT-1B and MT-4 expression was undetectable. Microarray analysis was also used to investigate MT gene expression in a human corneal epithelial cell line exposed to $ZnSO_4$ [87]. MT-1 group, in particular MT-1B, -1E, -1F, -1G, -1H, and -1X, were highly induced in corneal epithelial cells in the presence of zinc.

Regional (superior-inferior) variations in gene expression in the retina of C57BL/6J mice exposed to hyperoxia for 14 days were examined using microarray technique [88]. Relatively few genes were differentially expressed between the inferior and superior retina in normoxic conditions, but many immune-, cell defence-, and inflammation-related genes were identified as differentially expressed in hyperoxic conditions, and higher number of genes showed altered expression in response to hyperoxia in the inferior retina, which is known to be more vulnerable to hyperoxia. The expression of MT-1 and MT-2 were also more elevated in the inferior retina reflecting more tissue damage [88].

It is known that susceptibility of various inbred strains of mice to age-related retinal degeneration (ageRD) is different due to genes present in quantitative trait loci (QTL) on chromosomes (Chr) 6, 10, 16, 14, 18, 12, 13, and 8 [89]. Genes that are differentially expressed between ageRD resistant and sensitive strains were examined by microarray, and then genes that are localized to the Chr 6 and Chr 10 QTL were further analysed. It was noted that, interestingly, the expression levels of MT-1 and MT-2 genes are higher in posterior eyecups of ageRD resistant mice comparing with sensitive ones suggesting a difference between the two strains in the capacity to respond to oxidative stress [89].

3.2.2.10 Others

To gain an insight into the pathogenesis of non-steroidal anti-inflammatory drug-induced small intestine injury, gene expression profiles in the intestinal mucosa of Wistar rats were investigated 24 hs after indomethacin administration [90]. It was found that MT-1A was down-regulated in the intestinal mucosa after application of indomethacin, but the treatment with rebamipide, which inhibited the small intestinal injury, could reverse the altered gene expression.

Endotoxin tolerance means a reduced capacity of a mononuclear cell to respond to endotoxin (LPS) activation after an initial exposure to this stimulus, probably to protect tissues from hyperinflammation [91]. Gene responses in mononuclear cells during endotoxin tolerance were examined by microarray. It was shown that metallothioneins (MT-1H, MT-1F, MT-1A, MT-1X, MT-1E, and MT-2A) were strongly up-regulated in endotoxin tolerance [91].

4. Conclusions

Metallothioneins could be considered as promising prognostic/predictive biomarkers of oncology diseases, but the changes in their expression were also found in diabetes, cardiovascular diseases, psychiatric disorders, vision impairments and others (Table 1). The precise detection of low metallothioneins concentrations and their isoforms is necessary for understanding the connection between the metallothioneins quantity and isoforms and size, localization and type of cancer. This information could be useful for well-timed therapy and

could thus increase the chance to survival. Microarray chip appears as good possibility for studying isoforms' expression. In addition, microarrays allow investigating the gene expression differences and alterations in their network context thereby providing new data about functions and initiating biologically significant experimental studies.

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Conflict of Interest

The authors declare no conflict of interest.

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Captions for Figures

Figure 1

Summary of MT functions in human diseases.

Figure 2

Comparison of different requested steps for various microarray detections. (**A**) Describes two cell populations (diseased and control sample), RNA extraction, cDNA preparation and labelling by Cy5 (red) or Cy3 (green) fluorochrome, purification, targeting and fluorescence detection. (**B + C**) Describe steps needed for microarray detected by electrochemistry, (**B**) diseased and (**C**) control sample. Microarray probes are hybridized to biotin-labelled targets. The HRP-streptavidin conjugate binds to biotin, and enzymatic oxidation of the electron

donor substrate then occurs. The approach is based on the detection of redox active chemistries (horseradish peroxidase (HRP) and the associated substrate 3,3',5,5'–tetramethylbenzidine (TMB)) proximal to specific microarray electrodes. The detection current is generated due to electro-reduction of the HRP reaction product.

Figure 3

Schematic illustration of tissue microarrays fabrication and processing.

Figure 4

Scheme of a microarray-based experiment. After cells treatment or tissues sampling, RNA is extracted and/or converted to cDNA and hybridized with immobilized probes. In case of tissue microarray, the tissue cores are embedded into a paraffin block, sliced using a microtome and subjected to multiple (immuno)histochemical staining. The up/down-regulated expression of candidate proteins is validated by independent techniques.

Table 1. Summary of MT isoforms detected by microarray in different samples. Abbreviations: a – ageing, cd – cardiovascular diseases, d – diabetes, dt – drug toxicity, h – hepatotoxicity, n – nephrotoxicity, o – obesity, os – osteoporosis , p – psychiatric disorders, t – tumour, vi – vision.

Sample	Analysed molecules	Regulation of MT	References	Disease
Cell	cDNA	*MT-1B, MT-1K, MT-2A*	[48]	t
		MT-1X	[49]	t
	RNA	*MT-1A, MT-1B, MT-1E, MT-1G, MT-1H, MT-1M, MT-2A*	[50]	t
		MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1J (pseudogene), *MT-1M, MT-1L* (gene/pseudogene), *MT-1X, MT-2A*	[57]	t
		MT-1E, MT-1G, MT-1H, MT-1M, MT-1X, MT-2A	[59]	t
		MT-1B, MT-1E, MT-1G, MT-1H, MT-1L, MT-1X, MT-2A	[40]	t
		MT-1G, MT-2A	[64]	t
		MT-1G, MT-1H, MT-1L, MT-1M	[65]	t
		MT-1E, MT-1H, MT-1X	[72]	cd
		MT-1E, MT-1F, MT-1G, MT-1H, MT-1M, MT-1X	[73]	cd
		MT-1H	[75]	d
		MT-1F, MT-1M, MT-1X	[78]	h
		MT-1F, MT-1G, MT-1X	[79]	h
		MT-1A, MT-1E, MT-1F, MT-1G, MT-1X, MT-2A	[91]	dt
Tissue	cDNA	*MT-1F, MT-1H, MT-1X, MT-2A*	[60]	t
	RNA	*MT-2A*	[51]	t
		MT-1A, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, pseudogene *MT1-IP*	[54]	t
		MT-1G	[55]	t
		MT-1F, MT-1G, MT-1X, MT-2A	[58]	t
		MT-1B, MT-1E, MT-1G, MT-1H, MT-1L, MT-1X, MT-2A	[40]	t
		MT	[61]	t
		MT-1B, MT-1E, MT-1G, MT-1L, MT-1X, MT-2A, MT-3	[62]	t
		381 characteristically MT-responsive genes	[66]	t
		MT-1, MT-2	[68,69,76,88,89]	o, o, d, vi, vi
		MT	[92]	o
		MT-1	[70,81,84]	o, n, p
		MT-1X, MT-2A	[71]	cd
		MT-1E, MT-1G, MT-1M, MT-1X, MT-2A	[74]	d
		MT-2	[77]	d
		MT-3	[80]	h
		MT-1M	[82]	p
		MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A	[83]	p
		MT-1G	[86]	os
		MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1M, MT-1X, MT-2A, MT-3, MT-4	[87]	vi
		MT-1A	[90]	dt
	Tissue	MT-1H	[52]	t
		MT-1	[53]	t
		MT-1, MT-2	[56,63,85]	t, t, a

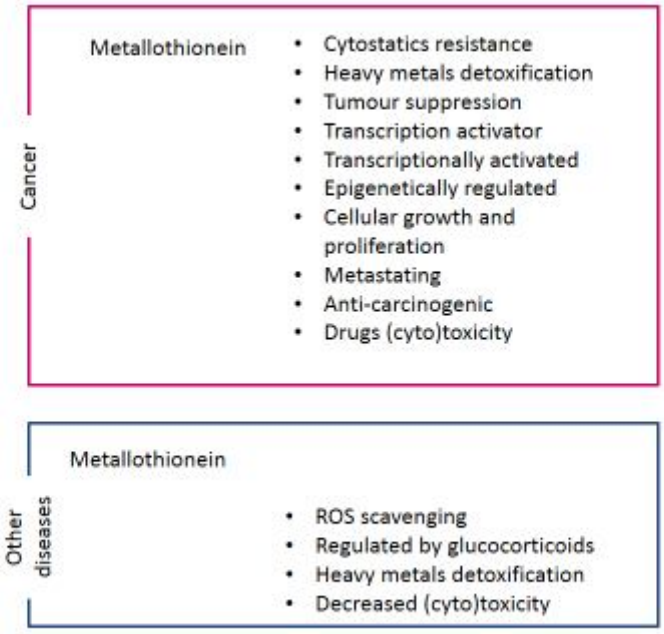

Figure 1

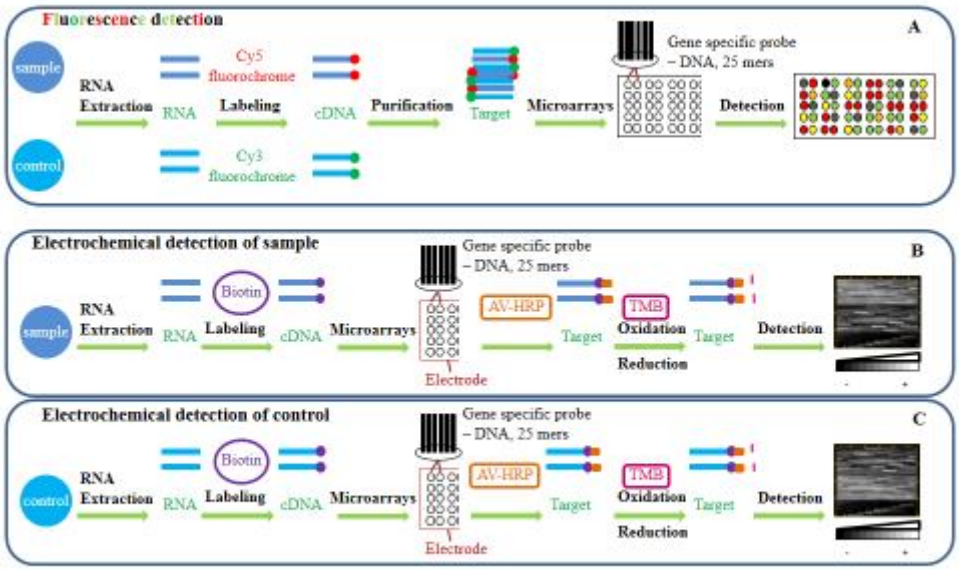

Figure 2

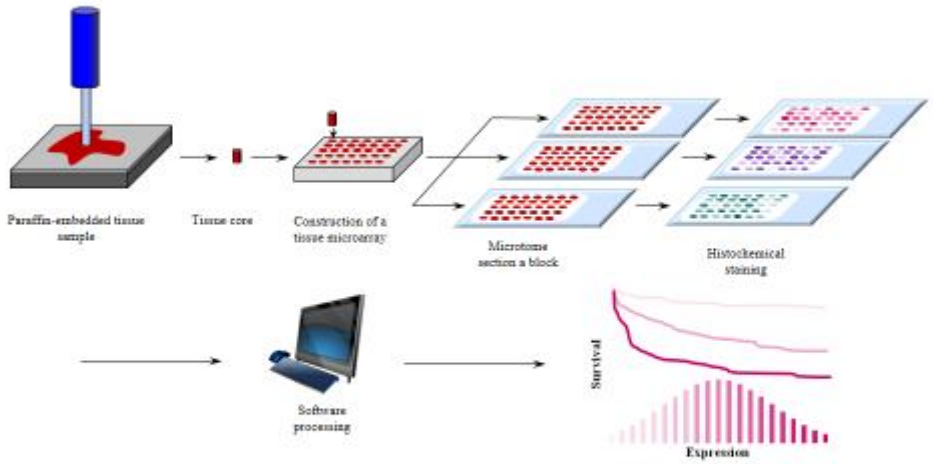

Figure 3

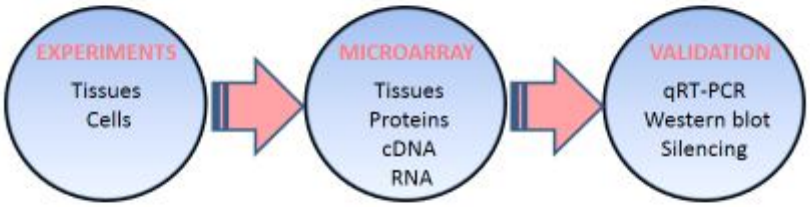

Figure 4