Metallothioneins in the lung cancer

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Abstract

Metallothioneins (MTs) are low weight proteins involved in several key cellular processes such as metal ions homeostasis, detoxification and scavenging of free radicals. Four groups of MTs are distinguished: MT-1, MT-2, MT-3 and MT-4. Regardless of the type, MTs are characterized by high content of cysteine, responsible for their biological properties such as binding of relevant zinc and copper ions, as well as toxic ions such as lead and cadmium. MTs were additionally shown to protect cells against oxidative stress damage and participate in differentiation, proliferation and/or apoptosis of normal and cancer cells. Many studies of different neoplasms showed association of elevated MTs levels with occurrence of chemo- and radiotherapy resistance and poor patients' outcome. In this review, we summarize and discuss the potential mechanism of action of metallotioneins in lung physiology and pathology. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 1, 1–10)*

Key words: metallothionein; lung cancer; non-small cell lung cancer; prognosis; proliferation

Abbreviations: AC — adenocarcinoma, ARE — anti-oxidant response element, CCT-LC — chronic cadmium-treated lung cells, EMT — epithelial-to--mesenchymal transition, FGF — fibroblast growth factor, GIF — growth inhibitory factor, GRE — glucocorticoid response element, HIF-1α — hypoxia-inducible factor-1α, HO-1 — heme oxygenase-1, iNOS — inducible nitric oxide synthase, LCC — large-cell carcinoma, LPS — lipopolysaccharide, MRE — metal response element, MT — metallothionein, MTF-1 — metal regulatory transcription factor, NSCLC — non-small cell lung cancer, OVA — ovalbumin, SCC — squamous cell carcinoma, SCLC — small cell lung cancer, SOD — superoxide dismutase, TGFβ — transforming growth factor β, VEGF-A — vascular endothelial growth factor A

Introduction

Lung cancer is reported to be one of the malignancies with the highest mortality both in Poland and worldwide [1, 2]. The majority of the diagnosed lung malignancies (84%) belong to the group of non-small cell lung cancers (NSCLC), and they are divided into three histological subtypes: squamous cell carcinoma (SCC), adenocarcinoma (AC) and large-cell carcinoma (LCC). Small cell lung cancers (SCLC) account for approximately 14% of the diagnosed cases [3]. Despite improvements in diagnostics and treatment, majority of the cases are diagnosed in advanced clinical stages with unfavorable disease course. It is estimated that only 16% of the patients survive longer than 5 years [1]. Taking into account high mortality and poor patients' outcome, studies on new prognostic and predictive markers of lung cancer are necessary.

Metallothioneins (MTs) are highly conserved low weight proteins (6–7 kDa) localized in the cytoplasm and cell nucleus. They were initially isolated from equine renal cortex and subsequently tested in other species [4]. A single MT molecule comprises 61 to 68 amino acids, which form two main domains designated as α and β [4, 5]. Studies on rodents enabled

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distinguishing four groups of metallothioneins: MT-1, MT-2, MT-3 and MT-4 [5, 6]. In humans, MTs are encoded by 17 genes, from which thirteen code for MT-1, two for MT-2 and one gene each codes for MT-3 and MT-4 [7–9]. Since now, ten genes were identified which encode functional MT proteins, MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3, MT-4, and seven genes coding for non-functional isoforms: MT-1C, MT-1D, MT-1I, MT-1J, MT-1K, MT-1L and MT-2B [7, 10]. Recently, a gene called MT-like 5 (*MTL-5*) and closely related in structure to other MTs was identified in mice testes. *MTL-5* encodes protein tesmin, differentially regulating meiosis in male and female cells [11]. Regardless of the type, MTs are characterized by high content of cysteine (up to one third of all amino acids), which thiol groups are responsible for their biological properties such as binding of relevant zinc and copper ions, as well as toxic ions such as lead, cadmium and mercury. Due to their properties, MTs participate in zinc and copper ions homeostasis and are capable of detoxifying the organism of other toxic metals [6]. MTs were additionally shown to protect cells against oxidative stress damage and participate in cell proliferation and/or apoptosis [4]. Although MT isoforms possess similar structure, their biological role may vary depending on cell type. Since now MT-1 and MT-2 isoforms are the best characterized and are ubiquitously expressed in normal, as well as cancer cells [4, 12, 13]. MT-3, isolated first from human brains affected by Alzheimer disease, was initially regarded as specific for nervous system; however, later studies demonstrated its expression also in cancer cells [14–18]. The MT-4 is so far the least characterized member of the MTs family and its expression has been mainly observed within the differentiating stratified squamous epithelia [8].

Metallothioneins MT-1 and MT-2

Metallothioneins of both these groups will be discussed together under the term MT-1/2, due to their high structural homology and functional, as well as biological, similarities. MT-1/2 are built of the α domain (comprising amino acids 31–68) and the β domain (comprising amino acids 1–30) [4]. The α domain comprises 11 cysteine residues capable of binding up to four divalent or six univalent metal ions, whereas the β domain comprising of 9 cysteine residues was shown to bind up to three bivalent and six univalent metal ions [19]. Due to the high structural homology of these conserved proteins, antibodies against their antigenic domains are not capable of distinguishing particular MT-1/2 members [20]. MT-1/2 expression

was shown in numerous studies to be induced by various agents such as heavy metals, steroid, free radicals, glucose, growth factors and cytokines. Among the above mentioned factors, zinc ions seem to be the main regulators of MT-1/2 expression in normal conditions [21–25]. Zinc ions regulate transcription of *MT-1/2* genes *via* activation of metal response element-binding transcription factor 1 (MTF-1), leading to its subsequent linking by its zinc finger domains with a specific DNA region — the metal response element (MRE) in the *MT-1/2* gene promoter [26, 27]. Besides, it was shown that metal ions other than zinc (*e.g.* cadmium and lead) may indirectly induce *MT-1/2* transcription. Due to their higher affinity to MT-1/2, cadmium and lead ions displace zinc ions from the MT-1/2 molecule resulting in elevated intracellular zinc concentration. Zinc ions bind next to the MTF-1, which, in turn, increases the expression of *MT-1/2* genes [28, 29]. Comparable mechanism was observed in regard to hydrogen peroxide (H_2O_2) that oxidizes MTs molecules, which results in the release of zinc ions [30, 31]. Moreover, the *MT-1/2* transcription was also shown to be initiated independently of MTF-1 through the activation of other *MT-1/2* promoter elements, such as antioxidant response element (ARE) during oxidative stress or glucocorticoid response element (GRE) in response to restrained stress conditions [32, 33].

MT-1/2 were shown to play important roles in various cellular processes. MT-1/2 regulate zinc ion homeostasis and may therefore regulate the activity of numerous transcription factors or zinc-dependent enzymes, *e.g.* metalloproteinases [34, 35]. Abundant lines of evidence point to the role of MT-1/2 in cell proliferation. Localization of MT-1/2 in proliferating cells depends on the phase of the cell cycle and was observed in nuclei, whereas in quiescent cells these isoforms are predominantly localized in the cytoplasm [36, 37]. Biochemical studies showed that while in the G1 phase the highest concentrations of MT-1/2 are found in the cytoplasm, during the G1/S phase a translocation of MT-1/2 molecules into the nucleus may be observed [37]. Furthermore, the highest concentrations of MT-1/2 molecules were detected in the S and G2 phases of the cell cycle, which underlines their role in cell division [37, 38]. Cytoplasmic-nuclear expression of MT-1/2 was noted in regenerating kidney [39] and liver [40] as well as proliferating basal and parabasal cells of stratified epithelia [41]. The role of MT-1/2 in the activation of cell proliferation seems to be supported by studies showing its positive correlation with the expression Ki-67 antigen in various tumors, *e.g.* breast cancer [42, 43], squamous and basal cell skin cancers [41, 44], adrenocortical cancers [45],

non-small cell lung cancers [19, 46], gastrointestinal stromal tumors [47] and sarcomas [48].

Many studies provided evidences for the key role of MT-1/2 in the protection of DNA against damaging effects of free radicals [49, 50], whether induced by radiation [51–53] or chemotherapeutic agents, *e.g.* cisplatin, bleomycin, etoposide, melfalan or irinotecan during ovarian, gastric, breast or prostate cancer treatment [13, 54–60]. Furthermore, MT-1/2 may also diminish the cytotoxicity of some alkylating drugs, like cisplatin and carboplatin by binding to their heavy metal ions [4]. In relation to its free radical scavenging function, MT-1/2 isoforms were shown to be potent anti-inflammatory mediators protecting cells against carcinogenic compounds [61, 62]. Furthermore, the stimulatory role of MT-1/2 on angiogenesis and collaterogenesis under stress conditions such as hypoxia was also presented [63, 64]. Elevated levels of MT-1/2 protein found in hypoxic conditions were shown to stabilize expression of hypoxia-inducible factor-1α (HIF-1α) [65]. It was also found that MT1/2 may induce the expression of proangiogenic factors, *e.g.* fibroblast growth factor (FGF), transforming growth factor β (TGF-β) and vascular endothelial growth factor A (VEGF-A) [19].

In spite of these apparently cytoprotective activities, in the majority of the studies MT-1/2 overexpression was associated with unfavorable prognosis in several malignancies, *e.g.* non-small cell lung cancer [66], intrahepatic cholangiocarcinoma [67], renal cancer [68], ovarian cancer [57], sarcoma [69] and melanoma [70]. However, in some tumors the prognostic significance of MT-1/2 expression seems to be limited, as in case of thyroid or laryngeal cancer [71, 72]; whereas in colorectal cancer high expression of MT-1 and MT-2 isoforms was associated with patient's favorable outcome [73]. Interestingly, the identification of particular isoforms of MT-1 and MT-2 revealed that not all of them were associated with patient's poor outcome. Some of them were shown to have cancer suppressive function. The MT-1G isoform was shown to be a potent oncosuppressor in papillary thyroid [74] and hepatocellular cancers [75], and MT-1F isoform — in colon cancer [76]. In contrast, the MT-2A isoform induces breast cancer cells proliferation and invasiveness *via* upregulation of matrix metalloproteinase-9 expression as well as the chemoresistance [35, 58, 77–79]. The overexpression of MT-2A in osteosarcoma cells suppressed the antineoplastic activity of the p53 protein and rendered the cells chemoresistance towards doxorubicin [80]. These data may indicate that despite high structural homology of particular MT-1 and MT-2 isoforms, they may play different roles in normal and cancer cells.

Metallothionein-3 (MT-3)

MT-3 was first described as GIF (growth inhibitory factor), because of its unique inhibitory role in neuronal outgrowth [14, 81, 82]. It was first isolated from human brains affected by the Alzheimer disease [14]. Its expression was initially thought to be restricted only to the nervous system — glial cells and neurons, but further research identified the presence of MT-3 also in other tissues, like testis, epididymis, seminal vesicles, prostate gland, ovary, uterus, heart, tongue, and stomach of rat and components of genitourinary tract in humans [16, 83–88].

MT-3 is built of 68 amino acids and, similarly to MT-1/2, organized into two domains, α and β. In spite of 70% structure homology, MT-3 molecule is characterized by the presence of a glutamate-rich hexapeptide containing a CPCP (Cys-Pro-Cys-Pro) motif localized near the C-terminus (amino acids 6–9). This unique peptide renders the structure of the MT-3 more nucleophilic and dynamic, and is probably responsible for the specific functions of MT-3 [81, 82]. The β-domain of the MT-3 molecule may bind only three bivalent ion metals, leaving two cysteine residues eligible. These residues may possibly participate in the interactions of MT-3 with thiol and disulfide groups of other proteins [89].

Although majority of studies suggest the uniform role of MT-1 and MT-2 isoforms in zinc ion homeostasis, scavenging of free radicals, proliferation, differentiation and apoptosis, studies analyzing the functions of MT-3 expression are not so consistent and bring divergent results. MT-3 was shown to exert protective effects, *e.g.* in *in vitro* models of epileptic brain and peripheral nerve injury [90]. However, the damage-promoting effects to neurons *via* highly increased intracellular zinc levels released from MT-3 molecules were also observed in *in vitro* studies [90]. Interestingly, also in other normal tissues and cancers frequently divergent results were observed in regard to the MT-3 function. The MT-3 immunoreactivity was found in normal human kidney [91] and in some cancer types; however, it varied strongly depending on the type of malignancy. Elevated expression of MT-3 mRNA, as well as MT-3 protein, was observed in prostate [16], breast [17, 88], and urinary bladder [18] cancers, whereas in gastric [83] and both types of esophageal cancer, *i.e.* squamous cell carcinoma and adenocarcinoma, expression of MT-3 mRNA was downregulated, but only in patients with methylated *MT-3* promoter [92, 93]. Taking into account that expression of MT-3 may additionally regulate lysosomal functions [90] and was shown to be induced in adipocytes under hypoxic conditions [94], further
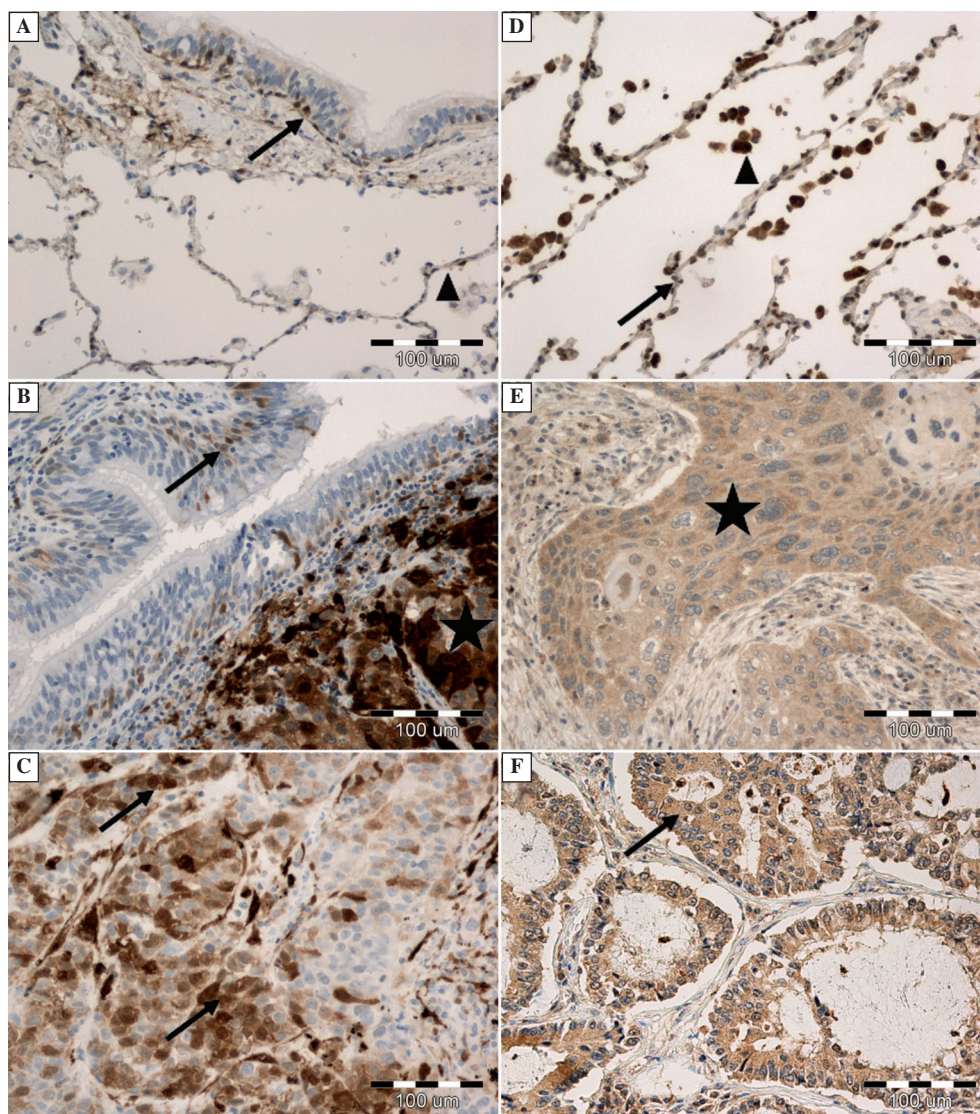
Figure 1. Immunohistochemical expression of MT-1/2 (A–C) and MT-3 (D–F) in normal lung and lung cancer. **A.** MT1/2 immunoreactivity is present in the nuclei of bronchial epithelial cells (arrow) and alveolar cells (arrowhead) of normal human lung; **B.** The presence of MT1/2 in the nuclei of bronchial epithelial cells (arrow) and in cytoplasm and nuclei of squamous cell carcinoma (SCC) cells (asterisk); **C.** MT1/2 presence in cytoplasm and nuclei of adenocarcinoma (AC) cells (arrow); **D.** MT-3 immunoreactivity in nuclei of alveolar cells (arrow) and alveolar macrophages (arrowhead) of normal human lung; **E.** MT-3 presence in the cytoplasm of SCC cells (asterisk); **F.** MT-3 in the cytoplasm of AC cells (arrow). The immunohistochemical reactions were performed by using commercial anti-MT-1/2 antibody (Clone E9, DakoCytomation, Denmark) and non-commercial, anti-MT-3 antibody (rabbit polyclonal antibody raised against GGEAAEAEAEKC peptide, Invitrogen, Carlsbad, USA). Normal human lung tissue was resected from non-malignant lung tissue adjacent to the primary lung tumor. Tumor tissue was obtained from patients during cancer resection

studies are needed to fully elucidate its role in carcinogenesis.

Role of metallothioneins in lung physiology and pathology

MTs were shown to be expressed in the cytoplasm and nuclei of normal pneumocytes and bronchial epithelial cells as well as cancer cells (Figure 1).

MT-1/2 were shown to protect lung tissue from several damaging agents such as bacterial endotoxin [95], ozone [96], carmustine (lipid soluble alkylating agent with high pulmonary toxicity) [97], ovalbumin (OVA) [98] and toxic metal ions such as cadmium [99–103]. It was demonstrated in human airway epithelial cells (BFAS-2B) that nickel increases MT-2A mRNA expression by mobilizing free intracellular zinc level [104]. MT-1/2 knock-out mice

were more susceptible to lung edema induced by intratracheal challenge with lipopolysaccharide (LPS) as compared with wild type controls [95]. The most prominent changes were observed in endothelial cells of pulmonary vessels and type I alveolar epithelial cells. In these cells, vacuolar degeneration and loss of basement membranes following LPS treatment were noted; however, these alterations were not found in wild-type control mice [95]. Moreover, higher levels of lipid peroxides in the lung suggested that oxidative stress could be important damaging factor in the lungs of LPS-treated MT-1/2-deficient mice [95]. Similar observations of the changes of lung ultrastructure and oxidative stress-related molecules were noted in MT-1/2 knock-out mice subjected to ozone treatment [96]. Moreover, expression of the oxidative stress-related molecules, such as heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), levels of 8-hydroxy-2'-deoxyguanosine, and nitrotyrosine, were significantly higher in the lungs of MT-1/2 knock-out mice as compared with the control group [96]. Although ozone and LPS exposure damaged lungs of MT-1/2 deficient mice more intensely, no changes in the expression levels of proinflammatory cytokines in bronchoalveolar lavage (BAL) could be observed in both these studies [95, 96]. Nevertheless, treatment of mice with ovalbumin led to much stronger increase of interleukin-1β levels in lungs of MT-1/2 knock-out mice as compared with wild type controls [98]. Similarly, 8-oxy-deoxyguanosine and nitrotyrosine levels were significantly higher in lungs of MT-1/2 knock-out mice which could indirectly indicate the protective function of MT-1/2 proteins against OVA-induced airway inflammation by oxidative damage suppression [98]. Taking into account the results of the above studies, besides MT-1/2 cytoprotective effects, its role of lung anti-inflammatory mediator and a possible marker of cell integrity can be implicated [61, 96].

The impact of MT-1/2 expression on tissue integrity and cell phenotype of pneumocytes and bronchial epithelial cells was also observed in *in vivo* and *in vitro* models following cadmium exposures; however, different mechanisms of action occurred in metal-intoxicated cells. Induction of MTs expression was noted following inhalation and intratracheal instillations of cadmium [99–103]. In lungs of Lewis rats pretreated with low dose of cadmium, increased MT-1/2 expression was found, which was associated with increased tolerance of the animals challenged with cadmium [101]. On the other hand, the cadmium adaptation of lung cells besides its protective effect for the lung was shown also to be a damaging factor for the lung cells [100]. A significant hyperplasia and hypertrophy of the alveolar epithelium in the lungs of cadmium-exposed

animals was observed [100]. Cadmium adaptation may also stimulate alveolar epithelial cells to proliferate and in this way contribute to the carcinogenic process [100]. Moreover, cultured cadmium-adapted alveolar epithelial cells were shown to possess reduced ability of DNA repair and were more resistant to apoptotic stimuli [100, 102]. To some extent in cadmium-pretreated alveolar cells, increased levels of MT-1/2 may sequester cadmium ions diminishing the cytotoxic effects of reactive oxygen species (ROS) [102]. However, at the same time these cells have reduced ability to repair the oxidative DNA damage once it occurred [102]. Recently conducted studies showed that epithelial lung cells (HPL-1D) chronically incubated with cadmium presented decrease in E-cadherin expression, a feature typical for cancer cells [105, 106].

According to Klaassen, intracellular MT-1/2 could diminish toxic effects of chronic cadmium-induced lung cells lesions such as pulmonary edema, hemorrhages, fibrotic changes and cancer [107].

On the other hand, in experimentally induced hyperoxia, MT-1/2 knock-out mice were characterized by decreased lung injury, such as reduced inflammation, interstitial edema and necrosis of airway epithelial cells, as compared with wild type controls [108]. Presumably, in the state of hyperoxia the lack of MT-1/2 is associated with observed increase of zinc ions level in the lung followed by compensatory increase in activity of antioxidant enzyme zinc-dependent superoxide dismutase (SOD) [108]. This may be a protective mechanism of airway epithelial cells against oxygen free radicals.

Metallothioneins' expression in lung cancer cell lines

Generally, the role of MTs in carcinogenesis has not been so far clearly defined, however, is seems that these proteins may exert only to some extent protective effects against heavy metal intoxication or ROS, implicated in the pathogenesis of some cancer types [109]. Once the protective potential of MTs has been reduced and neoplastic transformation took place, MTs may contribute strongly to tumor progression [6, 109]. The data provided in the preceding chapter clearly suggest that MTs are crucial in the adaptation of normal airway epithelial cells to inhaled cadmium and other toxic metal ions. However, the insufficient adaptative response could lead to malignant transformation of epithelial cells as has been schematically shown in Figure 2.

Recently, the human lung cancer cell lines A549 and H441 were shown to respond to higher levels of cadmium and zinc by increasing expression of
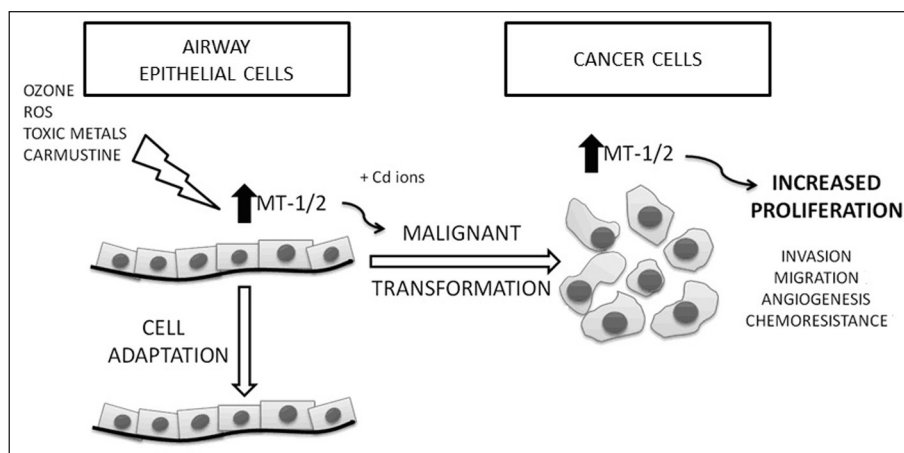
Figure 2. Possible action of MT-1/2 in normal airway epithelial cells and lung cancer cells. In response to noxious agents, an adaptive increase of MT-1/2 expression takes place in normal airway epithelial cells. The insufficient adaptive response could cause a malignant transformation of epithelial cells. Elevated MT1/2 expression in lung tumor cells is associated with increased cell proliferation and may have impact on invasion, migration, angiogenesis and chemoresistance of cancer cells

isoform MT-2A mRNA [110]. Moreover, in A549 cells an increase of another isoform, MT-1B, in response to nanosilver intoxication was observed [111]. Similarly, an increased level of MT-1/2 protein was also observed in A549 cells challenged with copper ions [112]. Concluding, these studies demonstrated that similarly to normal ones, also lung cancer cells respond to the increased level of metal ions by the upregulation of MTs expression. Most of the *in vitro* investigations were focused on determining the role of MT-1/2 in lung cancer cells' chemoresistance; however, they failed to establish any link between the MT-1/2 expression and resistance towards cisplatin and adriamycin [113, 114].

Role of metallothioneins' expression in lung cancer

Until now, only few studies analyzed the expression of MTs in lung cancer tumors in relation to clinicopathological data. Consistently across all studies, MT-1/2 levels were shown to be elevated in non-small cell lung cancer (NSCLC) cells as compared with normal epithelial airway cells [19, 115]. Moreover, the analysis of MT-1/2 proteins expression in lung cancer cases revealed their presence only in both types of NSCLC, squamous cell carcinoma (SCC) and adenocarcinoma (AC), and absence of MT-1/2 proteins in small cell lung cancer (SCLC) [115]. In addition, MT-1/2 expression was noted in the tumor cells as well as in the stroma of the NSCLC. The higher levels of MT-1/2 were observed in the SCC in comparison to AC type, but MT-1/2 levels did not correlate with the grade of the

tumors and lymph node metastases [115]. However, a study of Werynska et al. performed on high number of cases demonstrated the most pronounced expression of MT-1/2 in large cell carcinoma (LCC) as compared with AC [46]. Similarly to the observations of Theocharis et al., SCC presented significantly higher MT-1/2 levels as compared with AC cases. MT-1/2 immunoreactivity in cancer cells did not correlate with tumor malignancy grade, primary size, lymph node involvement or presence of necrosis [46]. To note, an increase of MT-1/2 immunoreactivity paralleling the increase of AC malignancy grade was observed [46]. However, the authors found a positive correlation of MT-1/2 expression with markers of proliferation (Ki-67 antigen and minichromosome maintenance protein 2 — MCM2) in cancer cells, concordantly to some previous studies [19, 66]. Despite the association of MT-1/2 expression with cellular proliferation markers, its immunoreactivity demonstrated no significant correlations with patient's survival and cancer prognosis [19, 46].

However, the molecular study of particular MTs isoforms in NSCLC and normal lung tissue revealed increased mRNA levels of *MT-1B*, *MT-1F*, *MT-1G*, *MT-1H*, *MT-1X* and *MT-3* genes, decreased *MT-1E* expression and no expression of *MT-4* isoform in NSCLC as compared with the normal lung tissue [20]. The high mRNA levels of *MT-1A* and *MT-1F* were associated with higher primary tumor size and highest malignancy grade [20]. Although MT-1/2 immunoreactivity had not have impact on NSCLC patients survival, cases with increased expression of *MT-1F* and *MT-2A* mRNA were characterized by poor outcome [20].

The potential role of MT-1/2 in the progression of SCLC is still poorly documented. It was demonstrated that high nuclear MT-1/2 immunoreactivity in this type of cancer cells was an independent predictive factor of patients' poor outcome [116].

A detailed analysis of immunohistochemical expression of MT-3 protein as well as expression of *MT-3* mRNA was also performed in NSCLC [15]. The presence of MT-3 protein was observed both in the cytoplasm and nuclei of cancer cells. A significantly lower nuclear MT-3 immunoreactivity was observed in cancer cells of the most undifferentiated NSCLC cases while the SCC cases were characterized by high nuclear MT-3 expression [15]. In this study, the SCC cases presented favorable prognosis. In all analyzed NSCLC cases low cytoplasmic MT-3 expression was noted in cases with higher primary tumor size. In contrast to MT-1/2, nuclear as well as cytoplasmic MT-3 expression seems not to affect cancer cells proliferation, as no correlation with the expression of the Ki-67 antigen was observed [15]. Interestingly, lower *MT-3* mRNA and cytoplasmic MT-3 expression were associated with poor patient outcome, but this finding was not confirmed in relation to MT-3 immunoreactivity in larger patients cohort [15].

The presented data suggest that the MT-1/2 and MT-3 expression in NSCLC seems to bear low prognostic and predictive role value, but may affect some processes during progression of this cancer, such as cancer cell proliferation. Further studies are needed to systemize and clarify the role of the MT family members in lung cancer.

Acknowledgments

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