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MINIREVIEW

# Clinicopathological Significance of Metallothioneins in Breast Cancer

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**Metallothioneins (MTs) are a family of metal binding proteins that play an important role in maintaining transition metal ion homoeostasis, redox balance in the cell and fundamental cellular processes such as proliferation and apoptosis. In humans, there are 4 groups of MT proteins which are encoded by 10 functional MT isoforms. In breast tissues, MT is primarily expressed in myoepithelial and malignant epithelial cells. Immunohistochemical studies have revealed that 26% to 100% of invasive ductal breast cancers express the MT protein. The MT-1F and MT-2A isoforms have been reported to be associated with higher histological grade in breast cancer, whereas higher MT-1E mRNA expression was found in estrogen receptor-negative tumors compared to their estrogen receptor-positive counterparts. A number of studies have shown that MT expression in breast cancer is associated with poorer prognosis. In addition, metallothionein expression may have a potential role in protecting the breast cancer cell from chemotherapeutic threats to survival.** (Pathology Oncology Research Vol 10, No 2, 74–79)

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## *Introduction*

Metallothioneins (MTs) are low molecular weight proteins of 6 to 7 kDa, with about 30% consisting of cysteine residues and no aromatic amino acids. The nomenclature for MT proposed by Kagi et al, define MTs as "polypeptides resembling equine renal metallothionein in several of their features".[1] They contain conserved sequences of cysteine residues juxtaposed with basic amino acids, such as lysine and arginine,[2] and these form metal-binding tetrahedral thiolate structures with special affinity for transition metals.[3]

The classification of MTs into families, subfamilies, subgroups and isoforms are based on sequence similarities and phylogenetic relationships.[4] In humans, MTs are encoded by a family of genes consisting of 10 functional MT isoforms which are located on chromosome 11q13.[5] The encoded proteins are classified into four groups, MT-1, MT-2, MT-3 and MT-4 proteins.[6,7] The functional genes of MT-1 encode MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H and MT-1X isoforms, whilst only one of the MT-2 genes, MT-2A, is functional. MT-3 is preferentially expressed in neural tissues[8] and MT-4 expression appears to be limited to squamous epithelial cells.[9]

## *Biochemical properties of MT*

All MTs have characteristic cys-x-cys, cys-x-y-cys, and cys-cys sequences, where x and y represent non-cysteine amino acids. Mammalian MTs are believed to bind a total of seven bivalent metal ions through thiolate coordination in two separate clusters.[10] To date, complete three-dimensional structures which have been elucidated for rabbit and rat MT-2,[11-13] confirmed the presence of two separate clusters, *viz*, beta-domain comprising amino acid residues 1 to 30 and three metal ions, and alpha-domain containing amino acid residues 31 to 61 and four metal ions. Because of its metal binding properties, metallothionein has been postulated to be involved in cellular homoeostatic control and regulation of trace elements. In mammals, zinc-metallothionein complexes appear to be the predominant form.[14] However, the ways in which zinc distribution in

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the cell is regulated and the mechanisms of zinc transfer from protein to protein are currently not well known.[15] It has been shown that zinc is easily displaced by other metals ions, such as lead and cadmium, by virtue of its low binding affinity with the apoenzyme.[16] Yet, the binding affinity *in vivo* appears to vary depending on the nature of stress experienced by the cell,[17] suggesting that transition metal ion homoeostasis is actively modulated, rather than a passive chemical process. It is also believed that the cysteine sulfur forming ligands to zinc can be reduced or oxidized with concomitant binding or release of zinc, respectively, and such oxidoreductive mechanisms may link metallothionein function with specific cellular signals.[18]

Metallothioneins can also serve as a redox buffer. The metal binding thiolate clusters have a low redox potential and are readily oxidized by cellular oxidants.[19] It has been shown that MTs can scavenge superoxide and hydroxyl radicals in a manner similar to thiol containing molecules, such as N-acetylcysteine and glutathione.[20,21] The binding of transition metals displaying Fenton reactivity (Fe and Cu) can also reduce oxidative stress. As a result, MT overexpression confers protection against free radical induced DNA damage,[22] and lipid peroxidation.[23,24]

The specific functional roles of each of the MT isoforms are not precisely known. Whereas mRNA of MT-1A, MT-1E, MT-1X, and MT-2A genes are expressed in normal prostate,[25] MT-1F, MT-1G and MT-1H mRNAs are additionally expressed in breast myoepithelial cells.[26] Interestingly, although metal response elements are present in the promoters of all MT genes,[27] not all MT genes are responsive to metal induction. MT-1A and MT-1E isoforms are up-regulated after exposure to cadmium and zinc,[28] whereas MT-1A and MT-1X are induced by arsenic[29] in the same cell line, and a different MT expression pattern is seen when different cell lines experience similar heavy metal exposure.[30] MT-1E, MT-1X and MT-2A isoforms were increased in PMC42 breast cancer cells that were resistant to copper and zinc toxicity.[31] Recently, a significant variant MT-1H isoform with amino acid replacements and notable changes in the secondary protein structure was reported in breast cancer cells.[26]

### *MT expression in human breast cancer*

MT expression is routinely visualized immunohistochemically using antibodies raised against the E9 epitope, which is conserved in both MT-1 and MT-2 isoforms. In a normal breast lobule that typically comprises bilayered ductules/acini (an inner epithelial layer and outer myoepithelial cells), strong nuclear and cytoplasmic MT immunopositivity was observed in myoepithelial cells and only rarely, in epithelial cells lining the large ducts.[32,33] Similarly, in other benign breast lesions such as adenosis, sclerosing adenosis and papilloma, only myoepithelial cells were shown to express MT.[34] Lobular cancer cells from in-situ or invasive tumors, showed weak to no expression of MT as well.[34,35] In contrast, a significant proportion of ductal breast cancers exhibited MT immunopositivity. Studies revealed that 26% to 100% of invasive ductal breast cancers express MT.[36-39] If a component of ductal carcinoma-in-situ was found in tumor tissues, the retained myoepithelial cells around these in situ islands were strongly highlighted immunohistochemically. In addition, MT expression was present in the in-situ cancer cells, with similar staining distribution and intensity to the surrounding invasive elements.[33]

The expression of different isoforms of MT mRNA in the breast cancer cell cytoplasm could also be demonstrated by in-situ hybridization on paraffin sections. Using RT-PCR on MT-expressing breast cancer tissues, the average quantity of MT-2A mRNA was found to be highest amongst the MT-1 and MT-2 isoforms and MT-1B mRNA was not detectable in all the samples.[38,40] Interestingly, MT-3 was also found to be expressed in 73% of breast cancers,[41] although it is not expressed in normal breast tissue.

### *Role of MT in breast carcinogenesis*

The potential role of MT in carcinogenesis has been well appraised by Cherian and co-workers, who were also the first research group to establish MT expression in human tumors.[42-45] As MT is known to influence tumor growth by affecting both cell proliferation and death, which are fundamental processes in carcinogenesis,[46,47] its role in tumors has attracted a lot of attention in recent years.

MT expression in many tissues of fetal mammals is higher than that seen in adults.[48] In human colonic cancer cells, it has been demonstrated that metallothionein expression is increased 2-3 fold in proliferating cell compartments compared to growth inhibited cells, and peak expression occurs during late G1 and G1/S transition phases.[49] The level of combined MT-1 and MT-2 expression in breast cancer tissue, and more specifically, the MT-2A isoform, correlate with increased proliferation indicated by Ki-67 immunopositivity.[38] It was demonstrated that overexpression of MT-2A in breast MCF-7 cells resulted in a 2-fold increase in cell multiplication,[50] whilst over-expression of MT-1E and MT-3 in breast cancer cell lines did not affect proliferative rate. In fact, in two cell lines studied, MT-3 over-expression resulted in growth inhibition.[51]

MT expression has been linked to reduced apoptosis in hepatocellular carcinoma[52] and nasopharyngeal carcinoma.[53] Although the relationship is not seen in breast cancer tissues,[38] interestingly, anti-sense down-regulation of MT-2A in MCF-7 cells was associated with both reduced cell growth and increased apoptosis with lower bcl-2 protein levels and decreased expression of c-myc mRNA tran-

***Table 1.*** **MT as a prognostic marker in breast cancer**

| *Reference* | *Country* | *Prognosis in relation to to high MT expression* |
|---|---|---|
| Ioachim et al., 2003[39] | Greece | Limited prognostic value |
| Vazquez-Ramirez et al., 2000[37] | Spain | Poor prognosis |
| Zhang et al., 2000[36] | China | Poor prognosis and higher histological grade |
| Oyama et al., 1996[64] | Japan | No correlation with prognosis |
| Goulding et al., 1995[66] | UK | Poor prognosis |
| Haerslev et al., 1995[63] | Denmark | Poor prognosis, axillary lymph node involvement, negative progesterone receptor status and higher histological grade |
| Fresno et al., 1993[32] | Spain | Poor prognosis, negative estrogen receptor status and higher histological grade |

scripts compared to controls.[51] It is possible that whilst MT expression may influence both proliferation and apoptosis, there are other more important factors that are called into play when apoptosis is triggered in breast cancer.[54]

The mechanism by which MT exerts its effects is not precisely known. MT was found to interact specifically with the p50 subunit of NF- B in MCF-7 cells,[55] and to inhibit the binding of NF- B to DNA following TNF activation.[56] The effect appears to be mediated by both MT-1 and MT-2 isoforms.[57,58] The possibility that MT might be able to interact with other proteins involved in cell proliferation and apoptosis was raised when MT-2A was also found to interact with esophageal cancer related gene 2 (ECRG2).[59]

There also appears to be a functional link between MT and the p53 tumor suppressor gene.[60] In the presence of zinc, MT facilitates normal functional p53 activity by zinc transfer between MT and p53, resulting in the maintenance of a DNA-binding conformation.[61] However, the transfer may be in the reverse direction under conditions of zinc depletion,[62] resulting in the disruption of the conformation of the DNA-binding domain and a phenotype similar to many mutant forms of p53. It has also been suggested that p53 and oestrogen-receptor may play a part in the expression and induction of metallothionein in human epithelial breast cancer cells.

### *Association of MT with pathological parameters and molecular markers of breast cancer*

MT expression in breast cancers has been studied in association with common clinico-pathological parameters used in breast cancer prognosis and other common oncogenes. High overall MT expression was consistently associated with increased tumor grade and more severe nuclear pleomorphism compared to the low MT expressing counterparts.[32,36,38,63,64] Some studies have also shown an inverse correlation between MT expression with estrogen receptor[32,64] and progesterone receptor content.[63,65] On the other hand, most studies showed no statistically significant association of MT expression with tumor size and with presence of lymph node metastasis at diagnosis,[38,39,64,66] although there is a numerical tendency for breast tumors of poorer stage to be more highly MT expressing.[38,39]

In breast cancer tissues, MT expression has also been studied in relation to the expression of tumor suppressor proteins (p53, pRb, Bcl-2), extracellular matrix components (type IV collagen, laminin), invasion- and tissue modeling-related genes (fibronectin, cathepsin D, CD44, matrix metalloproteinase-3), as well as growth factor receptors (c-erbB2, EGFR).[37,39,65,67] However, none of these biomarkers were associated with MT expression.

Looking into specific MT isoforms, Bay et al found that increased MT-1F and MT-2A mRNA were separately associated with higher histological grade, but not with patient age and lymph node status.[38,40] Higher MT-1E mRNA expression was found in estrogen receptor negative tumors compared to estrogen receptor positive ones.[68] However, there was no significant difference in MT-1E expression between progesterone receptor positive and progesterone receptor negative tumors.

### *MT as a marker of prognostication in breast cancer*

Higher MT expression in breast cancers has generally been shown to predict worse survival for patients (*Table 1*). Fresno et al.[32] found that patients with MT expressing breast cancers had decreased overall survival and shorter disease-free survival if the cancers were also estrogen receptor negative or lymph node negative. Other studies that included 72 to 478 patients,[36,37,39,63,66] have found worse prognosis associated with MT expression with the entire study population included in the analyses. A single study consisting of 92 patients found no statistically significant different in survival when the patients were stratified according to MT expression levels by univariate analysis.[64]

Multivariate analysis, including other clinico-pathological parameters, were reported only in a few studies,[37,63] and these showed that MT expression did not provide addi-

tional prognostic information with all other factors considered. This was probably due to the strong association of MT expression with other factors predicting poor prognosis (such as tumor grade) in the studies.

### *MT and chemoresistance*

Metallothionein has been extensively studied as a possible mediator of chemotherapy resistance.[69] In solid tumors treated uniformly with cisplatin-based chemotherapy, such as esophageal squamous cell carcinoma,[70] urothelial transitional cell carcinoma,[71] and small cell lung cancer,[72] metallothionein expression in the tumors have been associated with improved survival. It was felt that chemotherapy resistance to cisplatin is mediated, in part, by transfer of platinum from cisplatin to metallothionein, resulting in inactivation.[73] However, when ovarian cancer patients were treated with several chemotherapy regimes (some cisplatin-based), such a protective effect was not observed.[74,75] This suggests that the chemoprotective effect of metallothionein is probably regime specific.

Recent evidence suggests that metallothionein also reduces etoposide-induced apoptosis in lung and liver cancer cell lines, and the effect was increased with higher MT levels induced by pre-treatment with zinc or cadmium.[76] The mechanism by which metallothionein defer cell death from etoposide exposure is still not fully elucidated. However, little is known about the effect of metallothionein expression on the sensitivity of breast cancer cells to common chemotherapeutic agents used in the treatment of breast cancer. As drug resistance is a multifactorial phenomenon, the provision of direct and compelling evidence on the role of MT in chemoresistance in tumors is a difficult task.[77]

### *Conclusion*

Much remains to be learnt about the function of metallothionein in breast carcinogenesis and chemotherapy resistance, especially with regard to what role each of the isoforms performs in these processes. This may help in the development of a specific therapeutic agent that aims to correct the abnormal expression of metallothionein in breast cancers. Selective up-regulation of metallothionein in non-cancer tissues can also be explored further, so that existing treatment options may be utilized to greater effect.

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