Chapter-V
5.1. Introduction

Metallothioneins (MTs) are intracellular metalloproteins with significant distinctive aspects. These are ubiquitous low molecular weight (6-7 kDa), cysteine-rich and metal-binding proteins. It consists of 61-68 amino acid residues. Among them, 20 cysteine residues are (monovalent group 11 and divalent group 12) in a single polypeptide chain (Coyle et al., 2002; Vasak, 2005). MTs are known to play a significant role in metal homeostasis, detoxification, protection against free radicals, intracellular repair processes and differentiation (Kagi, 1991). MT proteins induced by various metal ions levels such as Zn, Cu, Cd, Hg, Co, Ni, and Ag etc. In addition, several factors are inducing MT such as heavy metals and other metals, glucocorticoids, cAMPs, interferon’s, interleukin-1, physical stress (food restriction and extreme cold), various drugs, herbicides, solvents, alkylating compounds, and ionizing irradiation (Miles et al., 2000). MT over-expression in certain types of tumors is thought to confer resistance to radiation and chemotherapeutic drugs (Moffatt and Denizeau, 1997). However, the exact mechanism of metallothionein is still not clear. It is reported that MTs play an important role as regulatory molecules in gene expression, homeostatic control of metals, adaptation to stress at the cellular level (Coyle et al., 2002; Vasak, 2005; Vergani et al., 2005). Earlier studies have been documented that, MTs play a key role in protection against metal toxicity. Since, MTs an essential protein in the cellular defense against Cd\(^{+2}\) toxicity but it provides much less protection against the lethality of the other metals such as Zn\(^{+2}\), Cu\(^{+}\), Fe, Pb\(^{+2}\), Hg\(^{+2}\) and As (Park et al., 2001).

MTs are participating in metal detoxification process in all organisms (Theocharis et al., 2003; Smirnov et al., 2005). They have the ability to store and release essential metals and maintain the low intracellular levels of free essential metals. MTs protect the cells by binding divalent heavy metals such as cadmium, lead, mercury and essential metals such as copper and zinc, which are toxic in surplus (Coyle et al., 2002; Smirnov et al., 2005). Other than heavy metals detoxification, MTs are found to be concerned with other protective mechanisms such as protecting the cells from oxidative stress, DNA damage, angiogenesis and apoptosis (Higashimoto et al., 2009). In human, MTs are classified as MT-1, MT-2, MT-3 and MT-4. These are encoded by 10 functional isoforms such as MT-1A, 1B, 1E, 1F, 1G, 1H, 1X, 2A, 3 and 4. The MT-1 and MT-2 have unique structure and binds to 7g atoms of divalent metals like zinc and cadmium. The MT-3 and MT-4 isoforms are normally found
in specialized cells. MT-3 is expressed mainly in neurons followed by glia. Whereas, MT-4 is tissue specific mostly present in differentiating stratified squamous epithelial cells (Theocharis et al., 2004; Vasak, 2005). These isoforms are expressed in a tissue specific pattern and may play a precious role in the different cell types.

Several reports have clarified the expression of certain specific isoforms in various human tumors. For example, MT-1 and MT-2 isoforms are expressed coordinately in most of the tissues, but exact mechanism of MT isoforms has not been elucidated. MT-2A has been found to be positively associated with the cell proliferation and apoptosis in liver tissues (Nagamine et al., 2005). Similarly, mRNA transcript of MT-2A has been reported to be over expressed compared to other isoforms in breast tumors. It has also been associated with estrogen receptor status, cell proliferation and histological grade tumors of breast cancer (Cherian et al., 2003). The MT-1F isoform expression has also been found to be associated with histological differentiation. Whereas MT-1E isoform expression is limited to estrogen receptor negative cells (Friedline et al., 1998). The positive expression of MT-3 is associated with a poor prognosis in breast cancer patients (Cherian et al., 2003). However, several studies have reported that, MTs are over expressed in breast tumor tissues at an early stage of tumor development. For this reason, the evaluation of metallothioneins may serve as an early biomarker of a carcinogenic process (Bay et al., 2006; Suzuki et al., 2007).

Therefore, the present study aims to evaluate the expression of selected metallothionein isoforms (MT-1F, MT-1E and MT-2A) in different clinical stages of breast cancer patients.

5.2. Results

In the present study, three metallothionein (MT) isoforms viz., MT1F, MT1E and MT2A were selected and their expression levels were determined by semi-quantitative RT-PCR in breast carcinoma tissues. It is well known that different MT isoforms possibly play different functional roles during development or under physiological conditions including metal ion homeostasis, pro-and anti-oxidant status of the cell and fundamental cellular processes and eventually regulates proliferation and apoptosis. Moreover, in clinical studies, MT is used as a promising prognostic biomarker in prospective studies. The selection of specific MT isoforms in the present study is based on the fact that MT-1F and
MT-2A isoforms have been reported to be associated with a higher histological grade in breast cancer, whereas higher MT-1E mRNA expression is found in estrogen receptor-negative tumors compared to their estrogen receptor-positive counterparts. The expression levels determined by using the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) technique which is well acknowledged sensitive approach in the determining the levels of mRNA expression. In the present study, the quantification of MT isoforms, a total of twelve individual pre and post menopausal subjects i.e., totally seventy two individuals were selected for the determination of MT expression levels. Similarly, MT levels were also quantified in non tumor breast tissue samples of respective individual subjects, during different clinical stages of pre-and postmenopausal breast cancer patients through RT-PCR technique. The equal amount of RNA was used for reverse transcription reaction. A total of 20µl of cDNA representing 500 ng of total RNA from both non tumor and tumor tissues are prepared using reverse transcription reaction and cDNA from both non tumor and tumor samples along with specific primers are used in the PCR reactions. The specific annealing temperature for each MT isoform is standardized using gradient PCR (Table 5.1). After completion of the PCR reaction, the amplicons are analyzed on 0.8% agarose gel electrophoresis and the alterations in the expression levels of MT isoforms are quantified by normalizing to the expression levels of housekeeping gene, GAPDH. Before confirmation, the expression levels are quantified by at least performing triplicate PCR reactions from each individual breast tissue of each MT subtype selected. Similarly, triplicate PCR reactions from each individual breast tissue are also performed for GAPDH which provide accuracy for normalization comparisons.

In the present study no significant percent changes were observed in the expression levels of GAPDH in normal and tumor samples of pre- and post-menopausal women at different stages of breast cancer (Fig.5.1). The percent change in the expression levels of GAPDH in different tumor stages I, II and III in the breast tissue of pre-menopausal women of tumor samples as compared to the expression levels of GAPDH in the normal breast samples (non tumor) of pre-menopausal women are 0.82, 0.86 and 0.85, respectively (Fig 5.1a). Whereas, the percent change in the expression levels of GAPDH in different tumor stages I, II and III in the breast tissue of post-menopausal women of tumor samples as
compared to the expression levels of GAPDH in the normal breast samples of post-menopausal women are 0.82, 0.91 and 0.84, respectively (Fig 5.1b).

The percent change was observed in the expression level of MT1F in non tumor and tumor samples of pre- and post-menopausal women during different stages of breast cancer patients (Fig 5.2). The percent change in the expression levels of MT1F in different tumor stages I, II and III in the breast tissue of pre-menopausal women of tumor samples as compared to the expression levels of MT1F in the normal breast samples of pre-menopausal women are 10.4, 16.7 and 23.4, respectively (Fig 5.2a). Whereas, the percent change in the expression levels of MT1F in different tumor stages I, II and III in the breast tissue of post-menopausal women of tumor samples as compared to the expression levels of MT1F in the normal breast tissue samples (non tumor) of post-menopausal women are 13.7, 9.17 and 16.2, respectively (Fig 5.2b).

The present study, the percent change was observed in the expression levels of MT isoforms in the breast tissue of pre- and post-menopausal women at different stages of breast cancer (Fig 5.3). The percent change was observed in the expression levels of MT1E in different tumor stages I, II and III in the breast tissue of pre-menopausal women as compared to non tumor breast samples of pre-menopausal women are 19.3, 26.9 and 25.5, respectively (Fig 5.3a). Whereas, the percent change was observed in the expression levels of MT1E in different tumor stages I, II and III in the breast tissue of post-menopausal women of tumor samples as compared to the expression levels of MT1E in the non tumor breast samples of post-menopausal women are 16.5, 25.9 and 24.8, respectively (Fig 5.3b).

The percent change was observed in the expression levels of MT2A in normal and tumor samples of pre- and post-menopausal women at different stages of breast cancer (Fig 5.4). The percent change in the expression level of MT2A in different tumor stages I, II and III in the breast tissue of pre-menopausal women of tumor samples as compared to the expression levels of MT2A in the normal breast samples of pre-menopausal women are 17.2, 26.7, and 24.9, respectively (Fig 5.4a). Whereas, the percent change in the expression levels of MT2A in different tumor stages I, II and III in the breast tissue of post-menopausal women of tumor samples as compared to the expression levels of MT2A in the non tumor breast samples of post-menopausal women are 19.2, 16.7 and 14.2, respectively (Fig 5.4b).
From the data, it suggests that, the expression levels of GAPDH mRNA in the breast tissue did not change in non tumor or tumor tissues stage and pre and postmenopausal of women. MT-1F isoform shows approximately 16, 12 fold mRNA expression in pre- and postmenopausal tumor tissues respectively over to non tumor tissues of women. Whereas, MT-1E shows approximately 21, 22 fold mRNA expression in tumor tissues respectively compared to relative non tumor tissues. Regarding MT-2A isoform shows 22 and 16 fold mRNA expression in pre- and postmenopausal breast tumors during all clinical stages respectively. On the other hand, as the tumor stage progressed from stage I to III, the expression levels of MT1F, MT1E and MT2A mRNA showed an increasing is observed in pre and postmenopausal tumor tissues, except, the expression levels of MT2A mRNA shows a declining trend from 19.2% (stage I), 16.7% (stage II) to 14.4% (stage III) in the post-menopausal women.

5.3. Discussion

Metallothioneins are highly conserved low molecular weight proteins. The main functions of MTs are binding, chelating and detoxification of various toxic substances (Smirnov et al., 2005; Vergani et al., 2005). MTs are involved in the homeostasis of the essential metal ions in the proper metabolism of an organism; they also take part in the detoxification of the tissues from toxic metals. MTs protects the tissue damage from reactive oxygen species, radiation, electrophilic pharmacological agents used in the cancer therapy, as well as protecting against mutagens (Smith et al., 2006; Thirumoorthy et al., 2007). MT has also been found to be associated with cell proliferation and apoptosis which has lead to the implication of MT in carcinogenesis. Thus, it suggests that the MT synthesis and distribution is a very important aspect in oncology, because these proteins not only indicate a defensive role, but also are responsible for the cell’s resistance to different pharmacological drugs (Zhang et al., 2000; Jin et al., 2004; Smith et al., 2006). Moreover, Metallothioneins are of great essential in breast cancer development as onco-proteins, promoting cell proliferation and apoptosis in several types of cancers (Hishikawa et al., 1999; Jayasurya et al., 2000), which has lead to the implication of MT in carcinogenesis. MT expression has been shown to be associated with a higher grade tumors, more aggressive breast cancer and poorer prognosis.
The expression of metallothionein isoforms MT-1F, MT-1E and MT-2A mRNA levels were observed in pre and post menopausal breast tumors compared to non tumors during different clinical stages. The percentage of expression levels were quantified by integrative density value (IDV). The expression rate increases along with stage progression in both pre and post menopausal breast tumors with selected MT isoforms in all clinical stages. This indicates that the MTs expression is closely associated with pre and postmenopausal tumors along with non tumor tissues. Earlier, it has been reported that MT-2A, MT-1E, and MT-1F mRNA expression in various cell lines and showed that the MT-2A isoform is highly expressed in all cell types (Schmidt and Hamer, 1986). Studies have documented that MT-1F gene expression is cell-type specific, regulated possibly by metals and glucocorticoids and related to DNA methylation and chromatin structure (Mididoddi et al., 1996). The expression of MT-1F mRNA levels is observed by RT-PCR analysis in different type of cell lines (Friedline et al., 1998). The MT-1F involved in cellular differentiation, it is a convoluted process, involving regulation of gene transcription, differential RNA processing, and translation as well as intra or inter cellular bimolecular regulation (Gilbert, 1997). In contrast, Yang et al. (1994) who also reported that no detectable MT-1F mRNA expression levels in their study of cisplatin treatment in squamous and small cell lung cancer cell lines. Therefore, it indicates that MT-1F isoform expression may be influence on tumor differentiation by means of several mechanisms. However, the exact role of MT-1F isoform in breast cancer is still unknown. Thus, additional research is needed to make clear the expression of the MT-1F isoform association with tumor aggressiveness in infiltrating (invasive) ductal breast cancer.

The MT-1E gene expression is cell-type specific (Schmidt and Hamer, 1986; Jahroudi et al., 1990) and plays a key role in metal binding process (Samson and Gedamu, 1998). The MT-1E expression is up-regulated by gene mutation in breast cancer tissues (Jasani et al., 1998) whereas, down-regulated in human prostate cancer (Garrett et al., 2000). In the present study, MT-1E expression was increased in both pre and postmenopausal breast tumors than non tumors. Studies of Cherian et al. (2003) reported that the MT-1E participates in the alternative processes that replace the function of estrogen. MT-1E isoform expression is associated with aggressive behavior in endometrial carcinoma tumors (McCluggage et al., 1999) and ER-negative human invasive ductal breast carcinoma (Jin et
This indicates that the MT-1E isoform expression has a prognostic significance in the cancer progression.

With regard to the role of MT isoforms in breast cancer, expression of MT-2A mRNA was observed to be significantly higher in pre and postmenopausal tumors compared with their respective non tumors in all clinical stage tumors. Tai et al. (2003) reported that an in vitro study of MT-2A isoform is unregulated in invasive breast cancer. The MT-2A expression may be associated with cell proliferation as evidenced by the observation that antisense down-regulation of MT-2A in the MCF7 breast cancer cell line induced cell growth arrest and apoptosis (Abdel-Mageed and Agrawal, 1997). Cui et al. (2003) also reported that MT might be able to interact with other proteins (NF-kB) involved in cell growth, proliferation and anti-apoptosis. In addition, MT-2A has also been shown to be implicated in metastasis through adhesion and migration of the breast cancer cells. It is possible either by aiding the transfer of essential metal ions between the interacting partners or directly binding to the proteins in the postulated pathways. The detection of the naturally occurring deletion mutant, MT-2A with lesser binding sites for divalent metal ions seems to support the former hypothesis as the MT-2A was shown to have a lesser potency in metastasis (Daina, 2009). Studies have also been reported that over-expression of MT isoforms are associated with good prognosis in breast cancer cell lines and tumors (Friedline et al., 1998; Ioachim et al., 2003). Thus, it could be a potential prognostic marker in breast cancer diagnosis and therapeutic plan.

From the data, it suggests that MT isoforms are closely related to breast tumor progression. Since MT isoforms are promising prognostic biomarkers in the prospective patient studies and may be validated to establish MT assays for use in clinical practice. The study on the role of MT isoforms in breast cancer may be useful not only in clarifying the clinical significance of MT isoforms but also to develop new strategies in enduring fight against breast cancer.
Table 5.1. Standardized annealing temperatures for MT isoforms

<table>
<thead>
<tr>
<th>PCR product</th>
<th>Denaturation</th>
<th>Annealing temperature</th>
<th>Optimal cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>95°C for 2min</td>
<td>52°C for 30sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>MT-1F</td>
<td>95°C for 2min</td>
<td>53°C for 30sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>MT-1E</td>
<td>95°C for 2min</td>
<td>55°C for 30sec</td>
<td>35 cycles</td>
</tr>
<tr>
<td>MT-2A</td>
<td>95°C for 2min</td>
<td>55°C for 30sec</td>
<td>35 cycles</td>
</tr>
</tbody>
</table>
Fig 5.1 RT-PCR analysis showing mRNA expression of GAPDH in the breast cancer patients

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), M-marker
Lane-1 (Non Tumor-GAPDH), Lane-2,3 and 4 - Tumor stage I, II and III GAPDH respectively
Pre-Postmenopausal, Post-Post-menopausal
Values in the parentheses are the percent change from the respective control (Non tumor)
Fig 5.2 RT-PCR analysis showing mRNA expression of MT-1F in the breast cancer patients

5.2a. MT 1F - Pre

5.2b. MT 1F - Post

Metallothionein Isoform-1F, M- marker
Lane-1 (Non Tumor- 1F), Lane-2,3 and 4 - Tumor stage I,II and III MT-1F respectively
Pre- Premenopausal, Post - Post-menopausal
Values in the parentheses are the percent change from the respective control (Non tumor)
**Fig 5.3 RT-PCR analysis showing mRNA expression of MT-1E in the breast cancer patients**

5.3a. MT 1E - Pre

5.3b. MT 1E - Post

**Metallothionein isoform-1E, M-marker**

Lane-1 (Non Tumor-1E), Lane-2, 3 and 4 - Tumor stage I, II and III MT-1E respectively

Pre- Premenopausal, Post - Post-menopausal

Values in the parentheses are the percent change from the respective control (Non tumor)
Fig 5.4 RT-PCR analysis showing mRNA expression of MT-2A in the breast cancer patients

5.4a. MT 2A - Pre

5.4b. MT 2A - Post

Metallothionein Isoform-2A. M - marker
Lane-1 (Non Tumor-2A), Lane-2,3 and 4 - Tumor stage I,II and III MT-2A respectively
Pre- Premenopausal, Post – Post-menopausal
Values in the parentheses are the percent change from the respective control (Non tumor)