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Clinicopathological study of metallothionein immunohistochemical expression, in benign, borderline and malignant ovarian epithelial tumors

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Summary. Metallothioneins (MTs) are a family of cystein-rich metal-binding proteins, which are expressed in normal cells during fetal and postnatal life but also in a variety of human neoplasms. MT expression in human tumors has been linked to resistance to anticancer drugs and differentiation and progression in some types of tumors. This study examined the immunohistochemical expression of MTs in benign, borderline and malignant tumors of ovarian surface epithelium and the possible correlations with clinicopathological parameters and survival. A total of 87 cases with diagnosis of ovarian surface epithelial tumors were included. Specifically, 21 cases of benign cystadenomas (11 serous and 10 mucinous), 14 borderline (low malignant potential tumors, 8 mucinous and 6 serous) and 52 cases of ovarian cancer were analysed.

Immunohistochemical expression of MT (cut-off level >10% of tumor cells) was clearly associated with malignancy. A statistically significant correlation was found between the expression of MT in cancer cases and benign tumors ($p<0.0001$) and cancer cases and borderline tumors $p= 0.003$. In cancer cases a difference was observed between grade I and III ($p=0.002$). There was no correlation of MT overexpression with survival in the small number of ovarian carcinoma patients where it was analysed. MT constitutes a marker that characterizes aggressiveness and a high malignant potential in ovarian epithelial tumors. In diagnostic problems MT may help distinguish between benign, borderline and malignant tumors.

Key words: Metallothionein (MT), Ovarian cancer

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Introduction

Metallothioneins (MTs) comprise a family of intracellular proteins of low molecular weight (6 to 10 kDa), with high cysteine content. They are present in animals, plants, fungi and cyanobacteria. In humans MTs are encoded by a group of genes, which are located on chromosome 16q13 (Hamer, 1986; West et al., 1990).

MT-I and MT-II represent the major isoforms that are known in mammals. Moreover, two other isoforms, MT III and MT IV, are found in specialized cells. MT III was first isolated as a growth-inhibiting factor (GIF) from brain neurons and MT IV in stratified epithelium (Brenner and Beattie, 1990). Structural studies have shown that the MT family of proteins has the ability to bind with the essential metals zinc (Zn) and copper (Cu) but also with the toxic metals cadmium (Cd) and mercury (Hg), and play a homeostatic role in the control and detoxification of these metals. MT is a potent antioxidant, protecting tissues in vivo and cells in vitro from various oxidative stresses (Miles et al., 2000). In humans, MTs are present in nuclear fetal liver cells but not in those of adults, in regenerating liver after partial hepatectomy, and in skin exposed to UVB irradiation indicating a physiological protective role in normal cells (Ktsujikawa et al., 1994, Anstey et al., 1996).

The possible role of MTs in cancer pathobiology has emerged, due to a variety of reasons. MTs are expressed in fetal neonatal life but also in different types of human tumors. This behavior of re-expression is similar to alpha-fetoprotein, which is used as a tumor marker in certain neoplasia. Its expression is restricted to embryonal and postnatal life but is re-expressed in some tumor cells. The presence of these proteins in tumor cells could be related to changes in proliferation or differentiation (Moffat et al., Cherian et al., 2003). MTs may increase tumor growth due to mitogenic effects and suppression of apoptosis and over-expression has been found to be associated with resistance to anticancer

drugs and radiotherapy (Cai et al., 1999; Haq et al., 2003; Tapiero et al. 2003). The protective role of MTs in oxidative stress and metal toxicity suggests that MTs may also have a functional role in tumor cell survival and growth (Cherian et al., 2003). MT expression has been associated with more malignant tumors and higher-grade tumors in some cases and with more differentiated lower-grade tumors in others (Jasani and Schimid, 1997).

Surface epithelial tumors originate from the celomic epithelium that forms epithelial glands and cysts (Auersperg et al., 1998) and are categorized by histopathological criteria for grading as benign, borderline and malignant tumors (Kurman, 1994). The prognosis for the malignant tumors unfortunately remains poor due to the advanced stage at presentation and the development of resistance to second line chemotherapy. To our knowledge, few studies have been made in ovarian cancer in order to analyze the significance of MT expression in relation to histopathological parameters and prognosis (Germain et al., 1996, Wrigley et al., 2000, Hengstler et al., 2001) and few which have examined the expression of MTs in the whole spectrum of surface epithelial tumors (Tan et al., 1999; Mccluggage et al., 2002).

In this study we examined the immunohistochemical expression of metallothionein in the full spectrum of ovarian surface epithelium tumors and the possible correlations with p53, proliferate indices (MIB1) and survival.

Materials and methods

Archival biopsy material of 87 patients, diagnosed with ovarian surface epithelial tumors between 1979 and 2003 were retrieved from the Surgical Pathology Department of the University Hospital of Ioannina. All patients had been surgically treated and received any further therapy at the University Hospital of Ioannina. The surgical treatment for benign tumors was simple cystectomy, and some of the benign cysts were removed also by oophorectomy, for borderline tumors conservative or radical surgery, and for malignant tumors radical surgery, which included hysterectomy with bilateral salpingo-oophorectomy and omenctomy. Patients with malignancy were referred to the Medical Oncology Department of the same Hospital and received chemotherapy according to running treatment protocols; all others were followed up by the gynecology dept.

Two pathologists blinded to clinical diagnosis reviewed the biopsies and two representative blocks from each case were selected for immunohistochemistry. All cases were analyzed by age, histological type, tumor grade and FIGO stage. Histological typing was performed according to the World Health Organization (WHO) criteria. This system considers both architecture and cytological features and carcinomas were graded as: GI (well differentiated), GII (moderately differentiated) and GIII (poorly differentiated). For tumor grading, the following criteria were used: tumor architecture, amount of solid tumor, nuclear pleomorphism, nucleus cytoplasmic ratio, number of nucleoli and mitoses (Kurman et al., 1994). For staging, the 1988 International Federation of Gynecology and Obstetrics (FIGO) recommendations were followed.

Overall, 21 cases of benign cystadenomas (11 serous and 10 mucinous), 14 borderline (low malignant potential tumors, 8 mucinous and 6 serous) and 52 cases of ovarian cancer were analyzed (Table 1). Regarding the 52 carcinomas, 31/52 (59.6%) were serous cystadenocarcinomas, 3/52 (5.8%) were mixed carcinomas, 3/52 (5.8%) were clear cell carcinomas, 4/52 (7.7%) were mucinous carcinomas, 4/52 (7.7%) were endometrioid carcinomas, 7/52 (13.5%) poorly differentiated carcinomas. In 25 of the 52 investigated cases of malignant tumors a complete follow-up of patients was available, including chemotherapy administered, response to the therapy, time to recurrence and survival. Regarding chemotherapy, 18 patients received the carboplatin-plaxitel combination and in 3 cases single-platinum chemotherapy was administered.

Immunohistochemistry

On two paraffin blocks selected from each case, we performed immunohistochemistry on 4μm tissue sections placed on poly-L-lysine-coated glass slides. Consequently, the sections were deparafinised in xylene and dehydrated. All sections were treated for 30 min with 0.3% hydrogen peroxide (in methanol) to endogenous peroxidase activity and then were incubated with primary antibodies. We used the method involving the vidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diamino-benzidine-H_2O_2 substrate for 5 min. The slides were counterstained in Harris' haematoxyline, dehydrated and mounted. To assess the specificity of the reaction, negative controls were included, and tumor sections

Table 1. Histology and incidence of the examined cases.

HISTOLOGY	PATIENTS	%
Benign tumors		
serous	11	12.6
mucinous	10	11.5
Borderline		
serous	6	6.9
mucinous	8	9.2
Malignant tumors		
serous cystadenocarcinomas	31	35.6
mucinous cystadenocarcinomas	4	4.6
mixed	3	3.4
clear cell	3	3.4
poor differentiated	7	8
endometrioid	4	4.6
Total	87	100

subjected to the standard procedure for incubation without the primary antibody. The antibody sources and dilutions are shown in Table 2.

Immunohistochemical evaluation

MT was mainly located in the cell cytosol where the protein is weaning, although sometimes there was combined nuclear and cytoplasmic staining (Bremmer, 1991) (Figs. 1-3). For the purpose of statistical analysis the cases were categorized for MT into four groups: 1 for negative, 2 lower than 10%, 3 between 10-50% and 4 higher than 50%.

Anti-p53 reactivity was evaluated only when brown nuclear staining was detected, and was scored as follows: 0 when less than 10% reactive cells, (1+) when the reactivity was between 10% and 25%, (2+) for 26% to 50% and (3+) when more than 51% cells were positive. Any case which scored at least (1+) was considered positive. The selection of this scoring system was based on the observation that when more than 10% of the tumor nuclei are stained with anti-p53, the highest correlation with the presence of structural mutations in the p53 gene is observed (Levine, 1993).

Anti-Mib1 reactivity was evaluated as positive only when epithelial nuclear staining was observed. For statistical analysis the cases were divided for MIB1 into two groups, (<10% and >10%) and for PCNA into three groups (<10%, 11-50% and >50%).

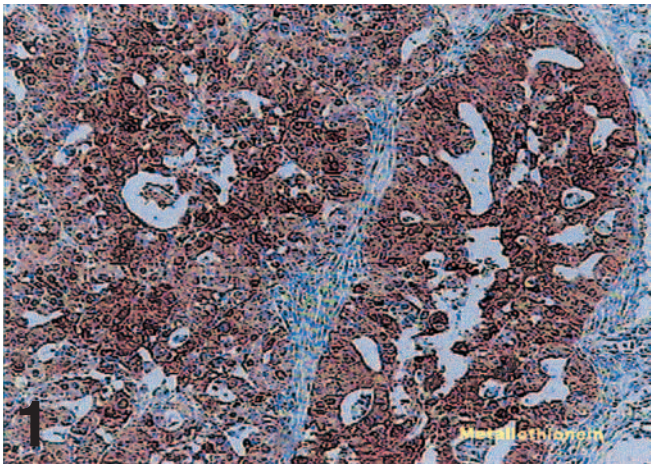
Statistical analysis

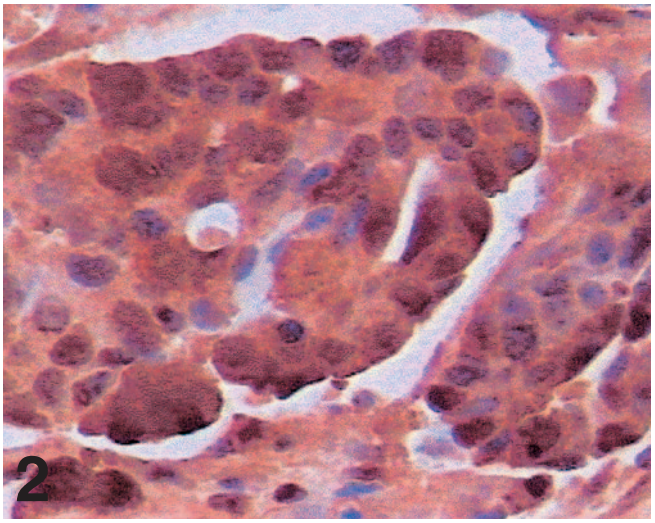
All data were statistically analyzed using the SPSS ver.10 statistical program. A non-parametric test of the Mann-Whitney U test type was used for the association of continuous variables. Survival was calculated using the Kaplan-Meir method and comparison of survival

Table 2. Antibodies used.

ANTIBODIES	SUPPLIER	DILUTION	INCUBATION TIME
Metallothionein,E9	Dako	1:50	Overnight*
MIB1	Dako	1:50	1 hour*
PCNA (PC-10)	Dako	1:50	1 hour
P53 (DO7, IgG2b)	Ylem	1:200	1 hour*

*: with microwave oven antigen retrieval





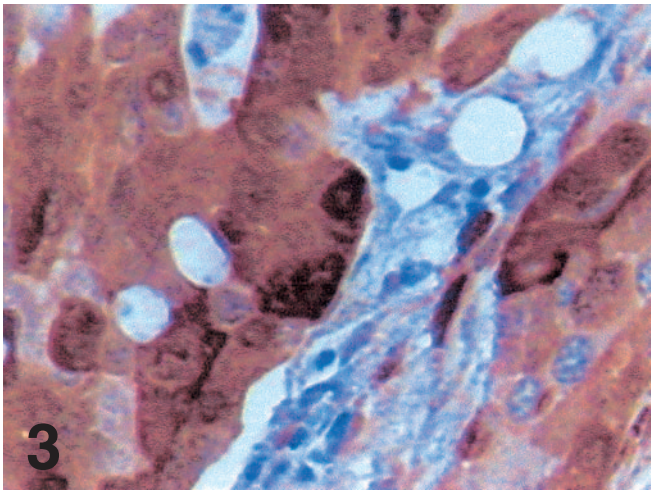


Fig. 1. Metallothionein immunohistochemical expression (ovarian cancer). x 100

Fig. 2. Metallothionein immunohistochemical expression (serous papillary cystadenocarcinoma). x 100

Fig. 3. Metallothionein immunohistochemical expression (mixed type carcinoma). x 100

rates was performed by the log-rank test. The Graphpad prism version 4 (Graph Pad Software, Inc, San Diego, CA) was used for survival analysis.

Results

Patient's ages at the time of the diagnosis ranged from 17 to 85 years old (median=58, mean=53). Patients were divided into three age groups: <45 years old, 45-55 and >55 years old.

MT immunohistochemical expression in cancer cases

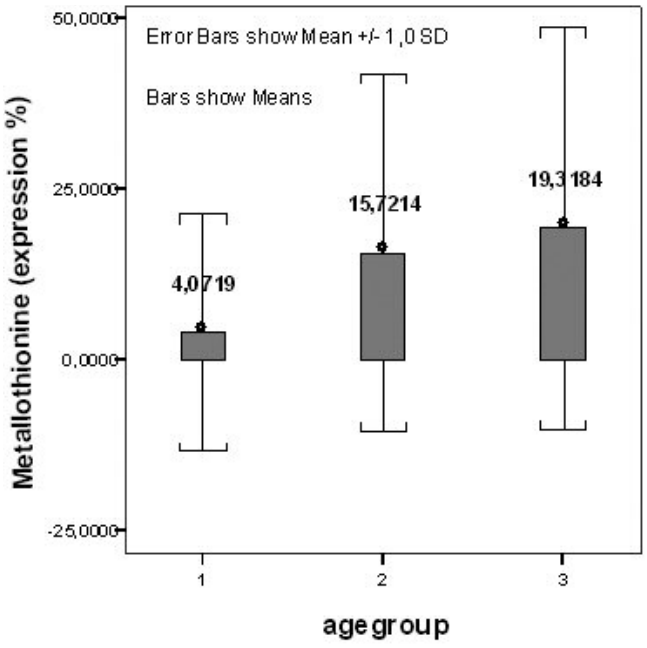
In carcinomas group, 52 cases, a statistically significant correlation between the expression of MT and age was observed, obviously related to the higher median age of patients diagnosed with malignant tumors (p=0.004), the immunohistochemical expression of MT increases with age (Graph 1).

The mean value of MT expression in cancer cases was 23.9%. 34.6% of the cases were negative (18/52), 23.1% of the cases (12/52) were lower than 10%, 11% (11/52) between 10 to 50% and 21.2% (11/52) were higher than 50%. A statistically significant correlation was found between the expression of MT and histological groups (Graph 2) between cancer cases and benign tumors (p<0.0001) and cancer cases and borderline tumors p=0.001.

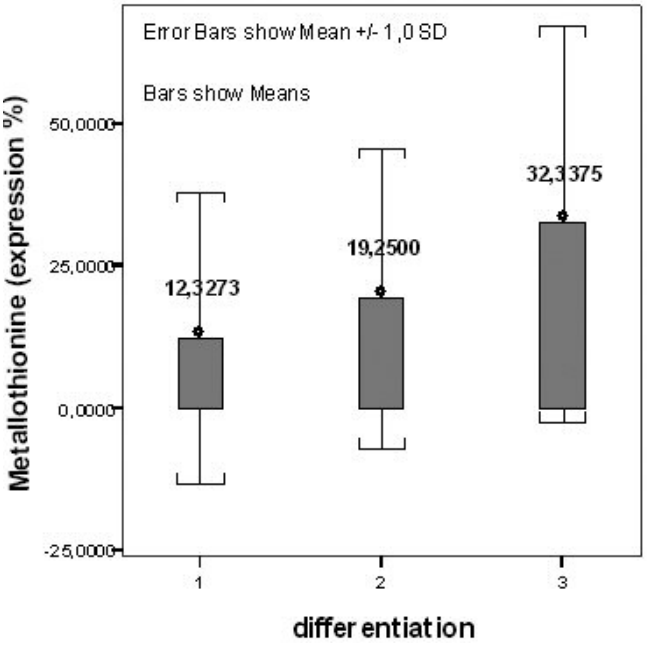
Regarding the histological grade, the mean value of MT expression was, in grade I: 9.5%, in grade II: 18.4%, and in grade III: 32.3%. There was a difference observed between grade I and III (p=0.002) (Graph 3).

No association between MT and stage was observed.

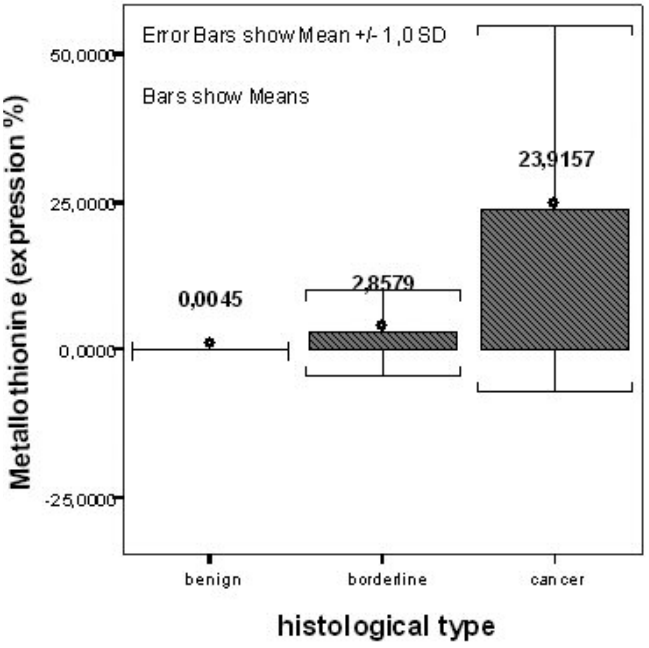
A statistically significant correlation was found



Graph 1. Expression of MT by age, in carcinomas group. Age groups: 1: <45 years old, 2: 45-55 and 3: >55 years.



Graph 2. Expression of MT by type.



Graph 3. Expression of MT by differentiation.

between MT and MIB1 ($p<0.0001$). No statistical correlation was found between MT and p53 expression. The immunohistochemical expression of MT was not found to be associated with a difference in survival rates in our patients.

MT immunohistochemical expression in borderline cases

In borderline tumours, the immunohistochemical expression of MT in the 85.7% (12/14) of the cases was negative and in 14.3% (2/14) of the cases was between 10-50% (Fig. 4). No statistical correlation was observed with the examined tumor markers.

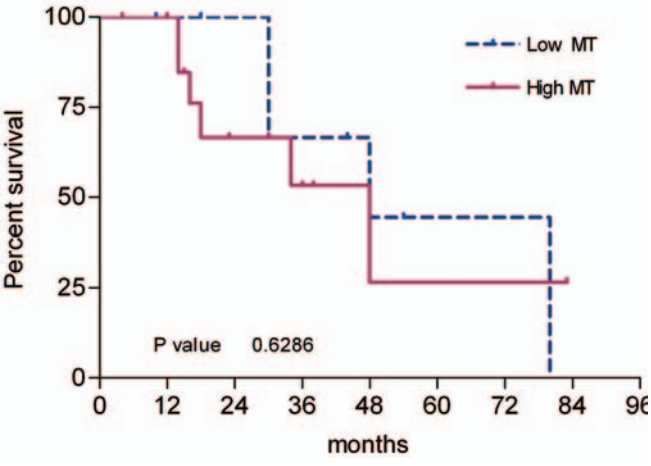
MT immunohistochemical expression in benign cases

In benign tumors 95.2% (20/21) was negative and 4.8% (1/21) was lower than 10%.

No statistical correlation was observed with the examined tumor markers.

Discussion

Abnormal cell growth, which could be the result of increased cell proliferation or from inhibition of cells undergoing apoptosis, is one of the steps that lead to malignancy. Altered levels of MTs can be expected in any situation where there is abnormal cell growth, such as cancer, considering that MT induction in normal cells is altered by a variety of physiological conditions such as changes in hormones, growth factors and accumulation of certain metals (Haq et al., 2003). The protective role of MT in oxidative stress and metal toxicity suggests that they may have a functional role in tumor growth and progression. MTs inhibit apoptosis and confer protection to neoplastic cells, which become resistant to antineoplastic drugs (Shimoda et al., 2003). This is the main problem in clinical practice for patients



Graph 4. Survival curve per MT expression of 21 patients with ovarian carcinoma treated with platinum based chemotherapy (Low = less than 10% stained cells, high = more than 10% stained cells).

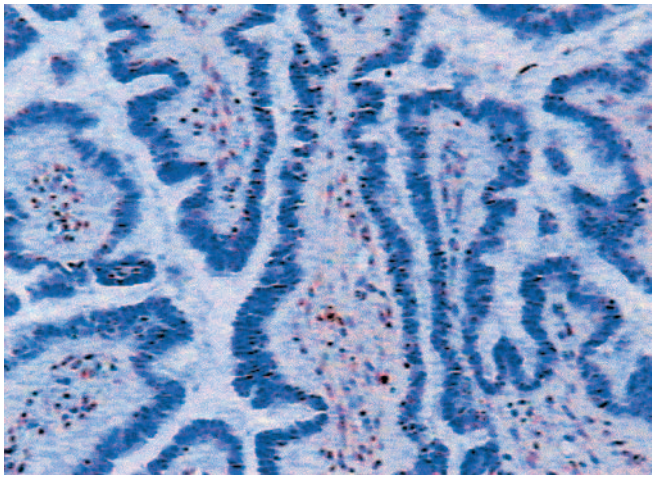


Fig. 4. Metallothionein immunohistochemical expression (borderline tumour). x 100

Table 3. Previous published data on MT expression in ovarian tumors.

AUTHOR AND YEAR	METHOD	No. PATIENTS-HISTOLOGICAL TYPE	PROGNOSTIC SIGNIFICANCE
Germain et al.1996	IHC	81 malignant tumors (pre-chemotherapy), 48 malignant tumors (post-chemotherapy)	No association with grade, stage, residual tumor after chemotherapy
Yi Tan et al.1999	IHC	12 benign, 14 borderline, 8 malignant tumors	Increased expression in malignant tumors is associated with worse prognosis
Wrigley E et al. 2000	IHC	59 malignant tumors (pre-chemotherapy), 21 malignant tumors (post-chemotherapy)	No association with survival, recurrence, grade, age, histological type
Hengstler JG et al.2001	IHC	151 malignant tumors 38 recurrences	Correlation of MT expression with grade. No association with survival, histological type
Mcc Luggage et al.2002	IHC	81 benign, 139 malignant tumors	A tendency toward higher expression in poorly differentiated tumors

with ovarian cancer. The majority of women, after an initial response to first-line chemotherapy, will eventually relapse to a more resistant status (Johnston and Gore, 2001).

In this study we observed that MT expression was higher in malignant cases than in benign and LMP tumors (Graph 1), which concords with the already published data (Tan et al., 1999, Mccluggage et al., 2002) and correlates with grade (Graph 2). A correlation with age was also noted, but only in the group of cancer cases in which MT is increasing with age (Graph 3), in agreement with Mocchegiani (Mocchegiani et al., 1997).

Surface epithelial tumors are categorized by histopathological criteria for grading, as benign, borderline or low malignant potential (LMP) and malignant tumors (Auersperg et al., 1998). It is unknown if this classification denotes a sequence to malignant transformation or whether it simply represents a spectrum of diseases. In this respect great interest has been aroused for the studying of LMP tumors. In a large study of Kurman and Trimble, which included 953 serous LMP tumors, only <1% demonstrated a malignant transformation (Kurman and Trimble, 1993). The reasons for the different expression of MT between borderline and malignant tumors remain unclear. The presence of MT in tumor cells could be related to changes in proliferation or differentiation (Moffat et al., Cherian et al., 2003). MT may play a role only in the development of malignant disease, which has not yet been clarified.

The possible correlation between p53 and MT has been investigated in different types of tumor cells. P53, the guardian of the genome, has the ability to cause cell cycle arrest in response to certain types of DNA damage, thereby allowing DNA repair to occur before cell cycle progression (Levine, 1993).

In vitro studies have shown that MT can modulate p53 conformation and transcriptional activity by chelation of zinc (Meplan et al., 2000). A relationship between metal induced induction of MT and the p53 status was observed in breast cancer epithelial cells with the wild type of p53 (MCF-7 cells) and mutated p53 (MDA-MB-231 cells). The p53 mutated cell lines were unable to induce MT or initiate apoptosis after the addition of cadmium or copper (Fan and Cherian, 2002). Zinc deficiency may reduce the ability of p53 to protect cells from carcinogenic compounds or conditions such as radiation; it appears that zinc depletion alters normal p53 expression. Zinc is crucial to maintain wild-type p53 conformation and DNA binding activity. Therefore, compromised cellular zinc status may possibly enhance the susceptibility of an organism to cancer by attenuating the tumor suppressive activity of p53 (Reaves et al., 2000). A statistically significant correlation between p53 and metallothionein expression was found in small cell carcinoma of the lung (Joseph et al., 2001). No statistical correlation between MT and p53 was found in urinary bladder carcinoma (Ioachim et al., 2001) and in malignant epithelium of the larynx (Ioachim et al., 1999). The present study was in agreement, with no statistical correlation observed between these two markers.

In a recent report, MT expression was correlated with ki-67 expression and a strong expression of MT and ki-67 was observed in lower grade (GII, GIII) colon tumors (Dziegiel et al., 2003). We also observed a strong correlation between MT and MIB1 in cancer cases

The previously published data concerning MT expression or significance in ovarian tumors is summarized in Table 3. The results are in conflict concerning the correlation with histopathological parameters and survival, but it is accepted that MT expression in neoplastic cells may protect them from antineoplastic drugs, especially from platinum compounds, and as a result confer drug resistance. The present study has shown that MT expression could be used in the discrimination between benign and LMP in malignant tumors, in agreement with Riskalla and Cherian (1997) who found that MT could differentiate between benign and neoplastic gastrointestinal infiltrates.

Based on our results we have concluded that MT expression in ovarian epithelial tumors can be considered a marker of aggressiveness and a high malignant potential of the tumors when over-expressed but is not associated with survival.

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