Immunoexpression of Metallothionein in Oral Squamous Cell Carcinoma

Rakesh Kumar Dumpala · Venkateswara Rao Guttikonda

Received: 18 July 2014 / Accepted: 28 February 2015 / Published online: 18 March 2015
© The Association of Oral and Maxillofacial Surgeons of India 2015

Abstract

Background and Objectives Metallothionein (MT) is a family of ubiquitous low molecular weight (7 kDa), intracellular (cytoplasmic/nuclear), cysteine rich proteins with high affinity for heavy metals, present in both normal cells and neoplastic cells. Increased expression of MT has been reported to be associated with poor prognosis in various tumours. The objectives of the present study were to compare the expression of MT in normal subjects and in patients with oral squamous cell carcinoma (OSCC), to correlate the expression of MT with respect to clinical staging of OSCC and to evaluate the expression of MT with respect to different histopathological grades of OSCC.

Methods Thirty cases of OSCC were staged clinically and graded histopathologically. Immunohistochemical method was used to detect the expression of MT in OSCC. The scores obtained were documented and compared statistically.

Results MT immunoreactivity was noticed in all cases of OSCC. A statistically significant difference was observed in immunoreactivity of MT between the normal and OSCC, and in different histopathological grades of OSCC ($p = 0.00001^*$). However, no statistical significance was found in a number of immunopositive cells in different clinical stages of OSCC ($p = 0.7573$).

Conclusion The MT immunoexpression increased from low grade to high grade OSCC. Hence, increased expression of MT may be related to poor prognosis in patients with OSCC.

Keywords Immunohistochemistry · Metallothionein · Oral squamous cell carcinoma · Prognosis

Introduction

MT belongs to a family of low molecular weight of about 6–7 kDa, non-enzymatic, cysteine rich proteins with high affinity for metal ions [1]. They consist of a polypeptide chain of 61–68 amino acids, of which around 30 % are cysteine residues. Cysteine forms a major component of MT structure due to their thiol (–SH) groups, through which metal ions are bound [2].

MTs make up a superfamily of proteins, with four isoforms identified as MT-1, MT-2, MT-3 and MT-4, encoded by a family of genes located on chromosome 16q13 [1–4]. MTs 1 and 2 are present in almost all the organs while MT-3 is found mainly in the brain and MT-4 in some stratified epithelia [1]. Since MT is present in most tissues and cell types in small amounts, it is generally considered as a “housekeeping” protein [5].

The principal and main function of MT is metal homeostasis and detoxification. MT has the capacity to bind both physiological (Zn, Cu, Se) [6] and xenobiotic (Cd, Hg, Ag) heavy metals through a thiol group of cysteine in its structure [7]. Apart from this MT is known to protect against reactive forms of oxygen, helps in the intracellular repair process along with growth and differentiation [8].

Various studies have demonstrated increased expression of MT in various human tumours such as larynx, pharynx, lung, kidney, chest, skin, ovary, prostate, bladder and pancreatic carcinomas compared to normal tissues [1]. However, very few studies have shown expression of MT in oral squamous cell carcinomas (OSCC).

R. K. Dumpala (✉) · V. R. Guttikonda
Department of Oral Pathology and Microbiology, Mamata
Dental College, Khammam 507002, Andhra Pradesh, India
e-mail: drrakeshbds05@gmail.com

V. R. Guttikonda
e-mail: mamatadental@yahoo.co.in

So, the present study was undertaken to evaluate the immunohistochemical expression of MT in OSCC and to correlate it with clinical stages and histopathological grades.

Materials and Methods

Thirty cases of OSCC and thirty normal subjects (controls) were included for the purpose of this study. After obtaining the informed consent from the patients and clearance from the ethical committee of our institution, the history and clinical findings of each patient was recorded. All the cases were clinically staged based on Tumour Node Metastasis (TNM) classification and graded histologically using Broder's grading system (1927). All the cases were confirmed by corresponding Haematoxylin and Eosin sections. The control group included thirty, apparently normal persons who visited our dental hospital for the extraction of impacted third molars. The soft tissue attached to the extracted tooth was fixed, processed and stained with a routine immunohistochemical procedure using MT primary antibody.

Immunohistochemistry

Formalin fixed, paraffin embedded tissue samples were studied by immunohistochemistry. Sections of 3 μm were obtained and mounted on 3-aminopropyltriethoxysilane coated slides. Sections were then dewaxed in xylene and hydrated through graded ethanol. Antigen retrieval was carried out with citrate buffer (pH 6.0) in the pascal chamber (Dako, CA, USA) for 30 s. Sections were immersed in 3 % H_2O_2 in methanol for 20 min for inhibition of endogenous peroxidase activity, and then blocked with 1 % bovine serum albumin in phosphate buffer saline (PBS) for 1 h. Slides were incubated with primary antibody against MT (clone E9, Dako, CA, USA). The primary antibody was diluted in PBS 1:50 and incubated for 1 h at room temperature. Subsequently, sections were incubated for 30 min with biotin-free horse radish peroxidase (HRP) enzyme labelled polymer (Dako, CA, USA). Diaminobenzidine was used as chromogen and sections were counterstained with Mayer's haematoxylin. Replacement of primary antibodies by non-immune serum served as negative control.

Sections of normal uterine cervix stained immunohistochemically using MT primary antibody served as positive controls.

Immunostaining Evaluation

The staining was considered positive when brown staining cells were observed, characterizing the presence of DAB in

the immunohistochemistry reaction. Random fields were chosen and the number of immunopositive cells was counted out of 1000 cells in each slide.

Statistical Analysis

The data obtained from the MT expression were subjected to statistical analysis. Student's *t* test was used to compare the number of immunopositive cells between the normal group and OSCC. ANOVA test was used to compare the number of immunopositive cells between different histological grades and clinical stages of OSCC.

Results

Within the normal group the MT immunoeexpression was observed in the basal and parabasal layers of the epithelium. Within the OSCC group MT immunoreactivity was sometimes observed to be restricted to the nucleus, sometimes to the cytoplasm and sometimes found in both the compartments.

A mosaic pattern was observed for MT immunoreactivity, with neoplastic cells showing high heterogeneity of staining, from negative to strongly positive. In well differentiated SCC the immunoreactivity of MT was predominantly restricted to the cytoplasm of the tumour cells and as the grade progressed to poorly differentiated, immunoreactivity was localised predominantly to the nucleus (Figs. 1, 2, 3, 4, 5, 6). Within the tumour islands, the immunostaining was observed in cells located at the periphery and at the centre of tumour islands, however MT immunostaining was restricted to the basal and parabasal layers wherever keratin pearls were found.

Fig. 1 MT immunoeexpression in well differentiated SCC ($\times 100$)

Fig. 2 MT immunoreactivity in well differentiated SCC ($\times 400$)

Fig. 4 MT immunoreactivity in moderately differentiated SCC ($\times 400$)

Fig. 3 MT immunoreactivity in moderately differentiated SCC ($\times 100$)

Fig. 5 MT immunoreactivity in poorly differentiated SCC ($\times 100$)

Table 1 shows a comparison of the expression of MT in normal subjects and in patients with OSCC. The mean number of immunopositive cells per 1000 cells in case of normal subjects and in patients with OSCC was found to be 100.50 and 618.67, respectively. Statistically significant difference was observed between normal subjects and in patients with OSCC with respect to the number of immunopositive cells (student's *t* test; $p = 0.00001^*$).

Table 2 shows the comparison of expression of MT with respect to different clinical stages of OSCC. The mean number of immunopositive cells in case of stage I ($n = 10$), stage II ($n = 8$), stage III ($n = 7$) and stage IV OSCC ($n = 5$) were 593.9, 681.38, 602 and 591.2, respectively. No statistically significant difference was observed between all the clinical stages of OSCC with respect to the number of immunopositive cells per 1000 cells counted in random fields (ANOVA test; $p = 0.7573$).

Table 3 shows the comparison of expression of MT with respect to different histopathological grades of OSCC. The mean number of immunopositive cells in grade I ($n = 12$), grade II ($n = 10$), grade III ($n = 8$) OSCC was found to be 426.33, 650.80 and 867, respectively. Statistically significant difference was observed between all the grades of OSCC with respect to the number of immunopositive cells per 1000 cells counted in random fields (ANOVA test; $p = 0.00001^*$).

Discussion

Over expression of MT is considered to have a potential against resistance to anticancer agents and radiation treatment [9]. This can be explained by the fact that MT has

Fig. 6 MT immunoreactivity in poorly differentiated SCC (×400)

Table 1 Comparison of expression of MT in normal subjects and in patients with OSCC with respect to the number of positive cells per 1000 cells counted in random fields

Groups	N	Mean	SD	T value	p value
Oral squamous cell carcinoma group	30	618.67	185.00	15.1941	0.00001*
Normal group	30	100.50	25.82		

Student's *t* test

* $p < 0.05$ —significant

ability to inactivate free radicals generated by some anti-neoplastic drugs like anthracyclins and bind to selected chemotherapeutics like cisplatin and carboplatin which results in inactive forms and impairing their capacity to kill neoplastic cells [10].

The worsening of prognosis in patients with increased MT expression was found only with cancers derived from ectodermal or paraxial or lateral mesodermal structures. OSCC which is ectodermal in origin has shown an inverse correlation between MT expression and overall survival of the patients [11].

Table 2 Comparison of expression of MT with respect to different clinical stages of OSCC by ANOVA test

TNM stage	Mean	SD	SE
Stage I	593.90	168.18	53.18
Stage II	681.38	217.43	76.87
Stage III	602.00	199.21	75.29
Stage IV	591.20	177.21	79.25
F value	0.3954		
p value	0.7573		

ANOVA test

$p = 0.7573$ —not significant

Table 3 Comparison of expression of MT with respect to different histopathological grades of OSCC by ANOVA test

Histo-pathological grades	Mean	SD	SE
Grade I	426.33	25.45	7.35
Grade II	650.80	45.49	14.39
Grade III	867.00	52.33	18.50
F value	284.8059		
p value	0.00001*		

ANOVA test

* $p < 0.05$ —significant

In the present study, MT immunoreactivity showed a statistically significant increase in OSCC group when compared to the normal group. Moreover, MT immunoreactivity was found to increase with increasing grades of OSCC; whereas, within the clinical stages of OSCC no statistically significant difference was observed.

In a study conducted by Muramatsu et al. [9], on the immunoreactivity of MT in head and neck carcinomas MT expression increased from well differentiated to poorly differentiated squamous cell carcinomas. Similarly, in our study MT immunoreactivity was increased from well differentiated to poorly differentiated OSCC.

In a study conducted by Szelachowska et al., immunoreactivity of MT, laminin, Ki-67 and Mcm-2 were studied in primary tumours with no metastasis to lymph nodes and primary tumours with draining regional lymph node metastasis. In patients with regional lymph node metastasis, a significantly higher MT expression was noticed when compared to patients with only primary tumours. Moreover, a predominant nuclear expression of MT was noticed in patients with lymph node metastasis when compared to patients with only primary tumours which showed predominant cytoplasmic expression [5]. This heterogeneity of MT expression in higher grades of tumour correlates with our present study.

Dutsch-Wicherek et al. have conducted a study on immunoreactivity of MT and vimentin in pharyngeal squamous cell carcinoma and its microenvironment and the results showed that MT immunoreactivity of MT was significantly higher in tumour islands and in the connective tissue stroma. The rationale for increased expression of MT in stroma is that MT expression might be evoked by the chronic inflammatory process developing in a tumour as a protective mechanism of healthy adjacent cells against immune mediated apoptosis. Hence, immunoreactivity of MT may be seen in the stromal fibroblasts. Cancer associated fibroblasts play an important role in tumour remodelling and so MT positive fibroblasts may cause remodelling of stroma and cause tumour spread [12].

Jayasurya et al. have conducted a study on expression of MT in nasopharyngeal carcinoma and its correlation with

tissue zinc levels. A linear relationship was observed between nuclear zinc levels and MT immunostaining. Zinc has the capacity to block apoptosis, as a result cell death is inhibited and abnormal proliferation of cells may lead to progression of tumour. Metallothionein being a metal binding protein may bind to zinc and cause progression of cancer [13].

In a study conducted by Lee et al. on immunoexpression of MT in areca quid chewing associated OSCC, high MT expression was associated with lymph node metastasis. This indicates the prognostic significance of MT in OSCC. Furthermore, no significant difference was observed in MT immunoexpression with respect to age, sex, T category and stage [14]. Similarly, in our study no significant difference was observed in different clinical stages of OSCC.

In a study conducted by Cardosos et al. [11] and Szelachowska et al. [15] on expression of MT in OSCC, high MT labelling indexes predicted shorter survival rate of the patients with OSCC. Furthermore, MT labelling index and clinical staging were found to be independent prognostic factors.

In a study conducted by Cardoso et al. [10] on expression of MT and p53 in OSCC, cases with concomitant expression of MT and p53 were significantly associated to faster deterioration of survival than other patients.

Studies on MT and Ki-67 expression along with tissue zinc levels in nasopharyngeal carcinomas (NPCs) and in soft tissue sarcomas, have shown a statistically significant correlation between MT and Ki-67 positivity and the grade of the malignancy. Tissue zinc levels were higher in NPCs when compared to benign nasopharyngeal tissues [16].

In a similar study on immunoexpression of MT and Ki-67 in selected soft tissue sarcomas (malignant fibrous histiocytoma, liposarcoma and synovial sarcoma), a positive correlation was observed between MT and Ki-67 expression, indicating that MT expression could be useful in defining the extent of malignancy and in prognosis of these tumours [17].

MT immunoexpression in odontogenic tumours such as ameloblastoma, have shown immunoexpression of MT in the peripheral columnar cells and in central polyhedral cells. The reason for immunoexpression of MT in ameloblastomas was thought to be that MT acts as a zinc reservoir for important proteases, such as matrix metalloproteases (MMPs) which help in the invasion of odontogenic neoplasms [1].

MT immunoexpression in oesophageal carcinomas, have shown an inverse correlation between its immunoexpression and overall survival of the patients [18].

These observations indicate that the multifunctional MT has a different expression in tumours with different origins and its overexpression is an indicator for poor survival in various cancers of the body.

Conclusion

Increased expression of MT is associated with poor prognosis of the patients as evident with increase in immunopositive cells with the progression of tumour to higher grades. However, further studies with larger sample are required to better characterize the role of MT expression in OSCC.

Conflict of interest None.

References

1. Ribeiro ALR, Nobre RM, Rocha GCMA, Lobato HIS, Junior SMA, Jaeger RG (2011) Expression of metallothionein in ameloblastoma. A regulatory molecule. *J Oral Pathol Med* 40:516–519
2. Dziegiel P (2004) Expression of metallothioneins in tumour cells. *Pol J Pathol* 55(4):3–12
3. Ebert MPA, Gunther T, Hoffmann J et al (2000) Expression of metallothionein II in intestinal metaplasia, dysplasia and gastric cancer. *Cancer Res* 60:1995–2001
4. Raleigh JA, Chou SC, Calkins-Adams DP, Ballenger CA, Novotny DB, Varia MA (2000) A clinical study of hypoxia and metallothionein protein expression in squamous cell carcinomas. *Clin Cancer Res* 6:855–862
5. Szelachowska J, Dziegiel P, Jelen-Krzeszewska J, Jelen M, Tarkowski R, Szytkowska B et al (2009) Correlation of metallothionein expression with clinical progression of cancer in the oral cavity. *Anti Cancer Res* 29:589–596
6. Szajerka A, Dziegiel P, Szajerka T, Zabel M, Windowski J, Grzebieniak Z (2008) Immunohistochemical evaluation of metallothionein, mcm-2 and Ki-67 antigen expression in tumors of adrenal cortex. *Anticancer Res* 28:2959–2966
7. Struys T, Krage T, Martens W, Theunissen E, Moreels M, Lambrechts I (2007) Metallothionein: a possible new marker for human dental pulp stem cells. *Eur Cells Mater* 14(2):131
8. Pontes HAR, Xavier FCA, da Silva TSP, Fonseca FP, Paiva HB, Pontes FSC et al (2009) Metallothionein and p-Akt proteins in oral dysplasia and in oral squamous cell carcinoma: an immunohistochemical study. *J Oral Pathol Med* 38:644–650
9. Muramatsu Y, Hasegawa Y, Fukano H, Ogawa T, Namuba M, Mouri K et al (2000) Metallothionein immunoreactivity in head and neck carcinomas; special reference to clinical behaviours and chemotherapy responses. *Anticancer Res* 20(1A):257–264
10. Cardoso SV, Silveria-Junior JB, Machado VDC, Paula AMBD, Loyola AM, Aguiar MCFD (2009) Expression of metallothionein and p53 antigens are correlated in oral squamous cell carcinoma. *Anti Cancer Res* 29:1189–1194
11. Cardoso SV, Barbosa HM, Candellori IM et al (2002) Prognostic impact of metallothionein in oral squamous cell carcinoma. *Virchows Arch* 441:174–178
12. Dutsch-Wicherek M, Lazar A, Tomaszewska R, Kazmierczak W et al (2013) Analysis of metallothionein and vimentin immunoreactivity in pharyngeal squamous cell carcinoma and its microenvironment. *Cell Tissue Res* 352:341–349
13. Jayasurya A, Bay BH, Yap WM, Tan NG, Tan BKH (2000) Proliferative potential in nasopharyngeal carcinoma: correlations with metallothionein expression and tissue zinc levels. *Carcinogenesis* 21:1809–1812
14. Lee SS, Yang SF, Ho YC, Tsai CH, Chang YC (2008) The upregulation of metallothionein-1 expression in areca quid

- chewing associated oral squamous cell carcinomas. *Oral Oncol* 44:180–186
15. Szelachowska J, Dziegiel P, Jelen-Krzeszewska J, Jelen M et al (2008) Prognostic significance of nuclear and cytoplasmic expression of metallothioneins as related to proliferative activity in squamous cell carcinomas of oral cavity. *Histol Histopathol* 23:843–851
 16. Jayasurya A, Bay BH, Yap WM, Tan NG, Tan BKH (2000) Proliferative potential in nasopharyngeal carcinoma: correlations with metallothionein expression and tissue zinc levels. *Carcinogenesis* 21(10):1809–1812
 17. Dziegiel P, Salwa-Zurawski W, Zurawski J, Wojnar A, Zabel M (2005) Prognostic significance of augmented metallothionein expression correlated with Ki-67 antigen expression in selected soft tissue sarcomas. *Histol Histopathol* 20:83–89
 18. Hishikawa Y, Koji T, Dhar DK, Kinugasa S, Yamaguchi M, Nagasue N (1999) Metallothionein expression correlates with metastatic and proliferative potential in squamous cell carcinoma of the oesophagus. *Br J Cancer* 81:712–720