MAD2 Expression in Oral Squamous Cell Carcinoma and its Relationship to Tumor Grade and Proliferation

CLARA RIZZARDI[1], LUCIO TORELLI[2], MANUELA SCHNEIDER[3], FABIOLA GIUDICI[4], LORENZO ZANDONA'[1], MATTEO BIASOTTO[5], ROBERTO DI LENARDA[5] and MAURO MELATO[6]

[1]*Unit of Pathology, Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy;*
[2]*Department of Mathematics and Earth Science, University of Trieste, Trieste, Italy;*
[3]*Unit of Pathology, ASS n.2 "Isontina", Gorizia, Italy;*
[4]*Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy;*
[5]*Unit of Odontology and Stomatology, Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy;*
[6]*Scientific Research Institute and Hospital for Pediatrics "Burlo Garofolo", Trieste, Italy*

Abstract. *Background: Defects in the cell-cycle surveillance mechanism, called the spindle checkpoint, might contribute to the chromosomal instability observed in human cancers, including oral squamous cell carcinoma. MAD2 and BUBR1 are key components of the spindle checkpoint, whose role in oral carcinogenesis and clinical relevance still need to be elucidated. Materials and Methods: We analyzed the expression of MAD2 in 49 cases of oral squamous cell carcinoma by immunohistochemistry and compared the findings with clinicopathological parameters, proliferative activity, BUBR1 expression and DNA ploidy. Results: MAD2 was over-expressed in 18 (36.7%) cases. Tumors with over-expression of MAD2 were associated with the progression of histological grade from well to poor differentiation ($p<0.001$), the extent of lymph nodes involvement (PN) ($p=0.0339$) and Ki-67 labeling index ($p<0.001$). Conclusion: MAD2 may be involved in oral carcinogenesis and may represent an important prognostic factor associated with a more malignant phenotype of oral squamous cell carcinoma.*

Chromosomal instability (CIN) leading to an aberrant chromosome number (aneuploidy) is a hallmark of cancer. A growing body of evidence suggests that defects in the cell-cycle surveillance mechanism, called the spindle checkpoint, might contribute to the chromosomal instability observed in human cancers. Molecular analysis of the genes involved in the spindle checkpoint has revealed relatively few genetic alterations, suggesting that the spindle checkpoint impairment frequently found in many human cancers might result from mutations in as yet unidentified checkpoint genes or altered expression of known checkpoint genes. A better understanding of this mechanism might provide valuable insights into CIN and facilitate the design of novel therapeutic approaches to treat cancer. The spindle checkpoint ensures accurate chromosome segregation during mitosis, inhibiting the anaphase-promoting complex or cyclosome (APC/C) to prevent the degradation of securin and cyclin B until all sister chromatids are properly attached to spindle microtubules. Key components of the spindle checkpoint include the evolutionarily-conserved MAD (mitotic arrest deficiency) and BUB (budding uninhibited by benomyl) proteins. In response to unattached or untense kinetochores, these proteins collaborate to inhibit APC/C and block anaphase onset (1).

Among the APC/C-inhibitory checkpoint mechanisms, MAD2 directly binds to the mitotic APC/C activator Cdc20, as part of a large complex that also contains BubR1 and Bub3 inhibiting its ability to activate APC/C (1). The gene encoding MAD2 has been analyzed in several different tumor types, showing evidence that mutations in the *MAD2* gene are actually infrequent (2). On the other hand, expression of MAD2 has been investigated in a number of human neoplasms (3-25). The majority of these studies have demonstrated that the expression of MAD2 is up-regulated in malignant tissues compared to their normal counterparts and correlates to several factors indicative of poor outcome, such as numerical chromosome abnormalities (8), high cell proliferation (16, 24), presence of aberrant mitotic figures (7,

Correspondence to: Clara Rizzardi, MD, Unit of Pathology, Department of Medical, Surgical and Health Sciences, University of Trieste, c/o Ospedale di Cattinara, Strada di Fiume 447, 34149 Trieste, Italy. Tel: +39 0403996239, Fax: +39 0403996247, e-mail: crizzardi@units.it

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17), more advanced stage (9, 18, 21, 25), higher histological grade (4, 6, 9, 16-18, 23, 25), presence of metastases (4, 18) and reduced survival (7, 9, 16, 18, 21, 25). These data suggest that over-expression of MAD2 might be a new tumor marker for predicting a more aggressive biological behavior of solid human neoplasms.

Like other solid tumors, squamous cell carcinoma of the oral cavity often exhibits chromosomal instability leading to aneuploidy. The mechanisms responsible for chromosomal instability in oral squamous cell carcinoma, however, are largely unknown. Spindle assembly checkpoint defects have been demonstrated in head and neck squamous cell carcinoma, including oral squamous cell carcinoma (26, 29). The *BUB1* gene has been investigated in oral cancer cell lines, thus suggesting that mutations in the spindle checkpoint genes are likely not involved in oral carcinogenesis (30). Conversely, spindle checkpoint malfunction seems to be related to an altered expression of spindle checkpoint proteins in oral carcinoma. Over-expression of Cdc20 has been associated to impairment of spindle assembly checkpoint and aneuploidization in oral cancer (27, 28). Cdc20 has also been identified as an independent prognostic marker of overall cancer-specific survival in patients with oral squamous cell carcinoma (29). Aurora B expression has been correlated with cell proliferation, histological differentiation and metastasis in oral squamous cell carcinoma, suggesting that it may be involved in tumor progression (31). Hannisdal *et al*. first examined the expression of BUBR1 and MAD2 in tonsillar carcinoma. They showed a strong correlation between reduced expression of BUBR1 and poor prognosis (32). On the contrary, in their study on oral squamous cell carcinoma, Lira *et al*. found that higher expression of BUBR1 was associated with lymph node metastases and shorter survival, and that it could be related to HPV status (33). We also analyzed the expression of BUBR1 in oral squamous cell carcinoma and, in agreement with Hannisdal *et al*., we showed that BUBR1 over-expression was significantly associated with a less advanced pathological tumor stage, possibly as a consequence of a less tendency to metastasize and to relapse, although recurrences seemed to occur earlier than in the group without over-expression of the protein (34).

To the best of our knowledge, the expression of MAD2 has never been investigated in squamous cell carcinoma of the oral cavity. In their series of tonsillar carcinomas, Hannisdal *et al*. found that MAD2 expression had no prognostic impact (32). Therefore, further studies are warranted to elucidate the role of MAD2 in oral carcinogenesis and its clinical relevance. In the present study, we analyzed the expression of MAD2 in oral squamous cell carcinoma by immunohistochemistry and compared the findings with clinicopathological parameters, proliferative activity, DNA ploidy and BUBR1 expression.

Materials and Methods

Patients and tissue samples. Forty-nine consecutive cases (38 men and 11 women) of oral squamous cell carcinoma were selected from the pathological files of the Unit of Pathology of the Azienda Ospedaliero-Universitaria "Ospedali Riuniti", Trieste, Italy. At the time of diagnosis, the patients' ages ranged from 44 to 86 years (mean=61 years). All tumor specimens were fixed in 10% buffered formalin and embedded in paraffin according to the standard protocol. Four micrometer thick tissue sections stained with hematoxylin and eosin were used for histopathological classification. Twelve cases were classified as well differentiated (grade 1), 20 as moderately differentiated (grade 2), and 17 as poorly differentiated (grade 3) squamous cell carcinomas. Pathological stages were pT1, pT2, pT3 and pT4 for 16, 21, 7 and 5 tumors, respectively. Regional lymph nodes were examined in 42 cases, 15 of which were pN0, whereas 10 were pN1, 15 were pN2b and 2 were pN2c.

Immunohistochemistry. Immunohistochemical staining was performed on the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ, USA). Briefly, a 5-μm thick section of each paraffin-embedded case was prepared. All tissue sections were de-paraffinized and subjected to antigen retrieval with Tris-EDTA at pH 9, then incubated for 1 h at room temperature with monoclonal antibody against MAD2 (1:50; BD Transduction, San Jose, CA, USA; product ID number: 610679) and BUBR1 (1:200; BD Transduction; product ID number: 612503) and for 16 min at 37˚C with pre-diluted antibody against Ki-67 (Ventana), respectively. Reaction products were visualized using the Ultra View Universal DAB Detection Kit (Ventana). A section of tonsil was used as positive control and sections incubated without the primary antibody served as negative controls.

MAD2 expression was calculated as the percentage of tumor cells with distinct nuclear staining in the most representative tumor areas in the entire section. At least 500 tumor cells were counted for each section. MAD2-positive cells in the mitotic phase were excluded from evaluation. The median level of expression of MAD2 was used as a cut-off to discriminate between high expression (above median) and low expression (below or equal to the median).

Intensity of cytoplasmic staining of BUBR1 was scored on a three-point scale as follows: –, no detectable expression; +, weak to moderate expression; ++, strong expression, comparable to that of cells in the germinal centers of normal tonsil. BUBR1 expression was calculated as the percentage of tumor cells with strong cytoplasmic staining in the most representative tumor areas in the entire section. At least 500 tumor cells were counted for each section. BUBR1-positive cells in the mitotic phase were excluded from evaluation. On the basis of findings from normal and dysplastic epithelium, BUBR1 was considered to be over-expressed when ≥20% of cells examined showed strong cytoplasmic expression.

The labeling index for the Ki-67 antigen was calculated as the percentage of tumor cells with distinct nuclear staining in the tumor areas most representative of the proliferative activity of the tumor in the entire section. At least 500 tumor cells were counted for each section. The tumors were divided into low and high proliferative activity groups, according to whether the Ki-67 labeling index was more or less the median value.

Flow cytometry analysis of DNA content. For the measurement of cellular DNA content by flow cytometry, nuclei suspensions

obtained from 30-μm thick sections of selected areas of each sample by pepsin digestion (Sigma Aldrich S.r.l. Milan, Italy; 1% in PBS at pH 1.5) were stained with a solution containing propidium iodide (P.I., 50 μg/ml in PBS; Igepal, 0.5%; RNAse, 75 KU/ml; Sigma Aldrich S.r.l.) overnight at 4°C. The samples were measured using a Coulter Epics Elite ESP flow cytometer equipped with a 488 nm argon ion laser and the Expo 32 Multi Comp Software (Beckman Coulter Inc., Brea, CA, USA). A lymph node diploid cell population sample was used as a reference standard for the calculation of the DNA index. The index was expressed by the relation between the aneuploid DNA content and the normal DNA value of diploid cells (DNA index=1). Cell cycle analysis was performed by the software program Multi cycle (Phoenix Flow System, San Diego, CA, USA).

Follow-up observations. Median follow-up after surgery was 41 months (range=0-142 months). During this period, 18 patients died due to unrelated causes, while disease recurred in 16 patients: local recurrence (11 patients), lymph node metastases (3 patients) and soft tissue metastases (1 patient); in one further patient, the disease recurred with both lymph node and soft tissue metastases. Ten patients died as a consequence of the disease.

Statistics. Statistical analysis was performed using the 2.6.2 version of the R Project for Statistical Computing (www.r-project.org). The relationships between categorical variables were assessed using the Fischer's exact test. The probability of survival was calculated by the Kaplan-Meier method with statistical differences evaluated by the log-rank test. For all statistical tests, $p \leq 0.05$ was considered significant.

Results

MAD2 stained the cells in the germinal centers of lymphoid tissue, which, if present, was used as inner control, and mitotic cells in both normal and neoplastic oral epithelium, as reported in the literature (35). Both in normal and neoplastic oral epithelium, MAD2 expression was mainly nuclear with an enhanced nuclear membrane (Figure 1). In some cases, positivity was limited to the nuclear membrane (Figure 2). In some tumors, additional cytoplasmic staining of MAD2 was observed, confirming previous observations (4, 6, 9, 11-13, 16, 18, 20, 24, 29, 32). Such a shift in the subcellular localization of MAD2 from the nucleus to also include the cytoplasm was especially observed in tumors with MAD2 over-expression (Figure 3). In the normal oral epithelium, MAD2 positivity was confined to the basal and parabasal layers. In the dysplastic epithelium neighbouring squamous cell carcinomas, MAD2-positive cells were more frequent and also detectable above the parabasal layer (Figure 4). MAD2 expression was observed in all cases of oral squamous cell carcinoma. In well-differentiated carcinomas, MAD2-positive cells were observed predominantly in the periphery of the tumor nests, while in poorly-differentiated carcinomas, MAD2-positive cells were present throughout the tumor nests, as previously described in relation to the expression in oral squamous cell carcinoma of other mitotic checkpoint proteins such as Aurora B (31) and BUBR1 (34) (Figure 5).

MAD2 was found to be over-expressed in 18 (36.7%) of the 49 cases.

A statistically significant correlation was found between MAD2 over-expression and the progression of histologic grade from well differentiation to poor differentiation ($p<0.001$). Moreover, MAD2 over-expression was statistically significantly correlated to the extent of lymph node involvement (pN) ($p=0.034$). Tumors with over-expression of MAD2 were also associated with a more advanced stage than those without it, but the correlation was not statistically significant ($p=0.146$). Furthermore, MAD2 expression was significantly correlated to Ki-67 labeling index ($p<0.001$).

There was no statistically significant correlation between MAD2 expression and the trends of cumulative and recurrence-free survival. No significant correlation was seen between MAD2 and BUBR1 expression.

Eleven cases failed in correct quantification of DNA content and had to be excluded. DNA aneuploidy was found in 15 (39.4%) of the remainder 38 cases. No statistically significant correlation was found between DNA ploidy and MAD2 expression. Distribution of MAD2 expression status and associations with general clinicopathological parameters are summarized in Table I.

We also analyzed the association between the C/N ratio of MAD2 expression and clinicopathological parameters, showing that a C/N more than 2 was statistically significantly correlated to Ki-67 labeling index ($p=0.007$) and also related to histological grade, but with no statistical significance ($p=0.1$).

Results concerning the expression of BUBR1 and its relationship to tumor stage and survival have been presented and discussed in a previous publication (34).

Discussion

In the present study, we investigated the expression of the mitotic checkpoint protein MAD2 in squamous cell carcinoma of the oral cavity by immunohistochemistry. Out of the 49 oral squamous cell carcinoma samples analyzed, about a third (36.7%) showed an increased expression of MAD2.

Interestingly, we have found that MAD2 over-expression was strongly statistically significantly associated not only with the progression of histologic grade from well to poor differentiation ($p<0.001$) but also with the highest values of Ki-67 proliferative index. A relationship between MAD2 up-regulation and poor differentiation has been previously observed in a number of solid neoplasms, such as colorectal carcinoma (6), bladder carcinoma (9), gastric carcinoma (14), hepatocellular carcinoma (16), soft tissue sarcoma (17) and osteosarcoma (18), ovarian carcinoma (23), endometrial carcinoma (25), but not in tonsillar carcinoma (32). Certain

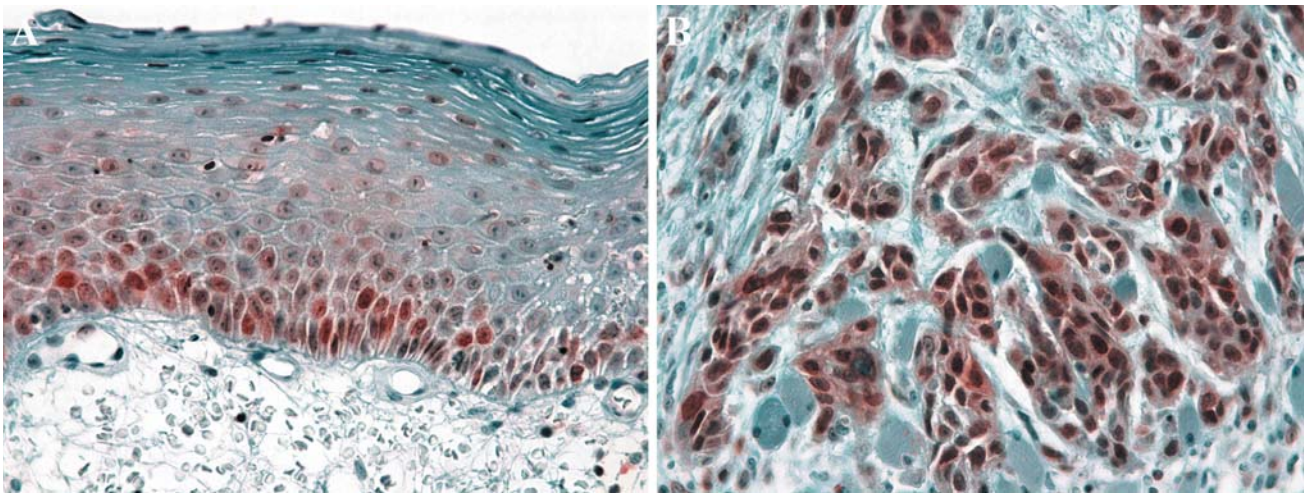

Figure 1. *Nuclear staining pattern of MAD2 expression in normal (A) and neoplastic (B) oral epithelium (MAD2 immunohistochemical staining, original magnifications, ×400).*

authors have also demonstrated an association between MAD2 over-expression and high Ki-67 labeling index in hepatocellular (16) and ovarian carcinoma (24), whereas in their study on tonsillar carcinoma Hannisdal *et al*. found that BUBR1 expression was significantly correlated to Ki-67 positivity, but MAD2 expression was not (32). A more advanced histological grade and a high proliferative index are well-known adverse prognostic factors in a variety of tumors, including oral squamous cell carcinoma. Despite the discrepancies between our results and those of Hannisdal *et al*. on tonsillar carcinoma, our findings suggest that over-expression of MAD2 may be involved in oral carcinogenesis and taken into account as a prognostic marker in squamous cell carcinoma of the oral cavity. In confirmation of this assumption, we also found that MAD2 over-expression was statistically significantly correlated to the extent of lymph node involvement (pN) (p=0.034). A relationship between MAD2 expression and metastatic potential has also been demonstrated in colorectal carcinoma (6), gastric carcinoma (4) and osteosarcoma (18). Moreover, we found a relationship between MAD2 up-regulation and advanced pathological stage, which is probably the consequence of the dependence between MAD2 expression and pN status. In this case, however, the p-value of 0.146 indicated that the association was not statistically significant, although this value most likely reflects some limitations related to the small size of the sample and might decrease to reach a significance level with a numerical increase of the series.

In the present study, the prevalence of DNA aneuploidy in oral carcinoma was 39.4%. No statistically significant correlation between DNA ploidy and MAD2 expression was found. At present, there are only two studies available that have

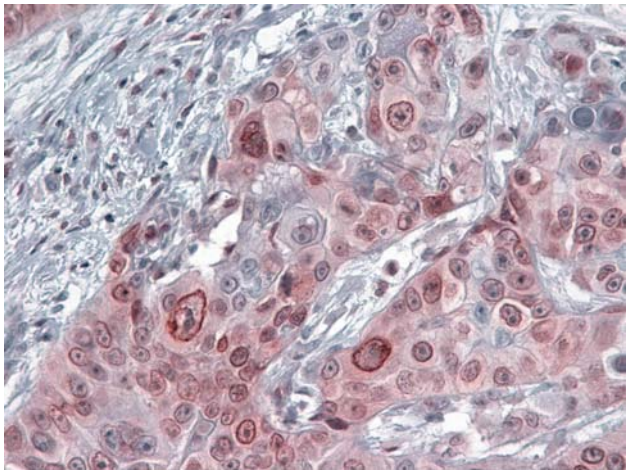

Figure 2. *MAD2 positivity limited to the nuclear membrane, original magnification, ×400).*

unsuccessfully attempted to find a relationship between the expression of MAD2 and ploidy status in gastric (3) and in pancreatic (20) carcinoma. These findings lead us to re-state that, also in oral cancer, aneuploidy might be due to inactivation or de-regulated expression of other components of the spindle-associated protein complex such as Cdc20 (27) and Aurora B (31) but not BUBR1, the expression of which was found to be independent of DNA content in our previous study on BUBR1 expression in squamous cell carcinoma of the oral cavity (34).

In our series, tumors with MAD2 over-expression had a tendency to show a shift in the subcellular localization of the protein from the nucleus to also include the cytoplasm. Fung

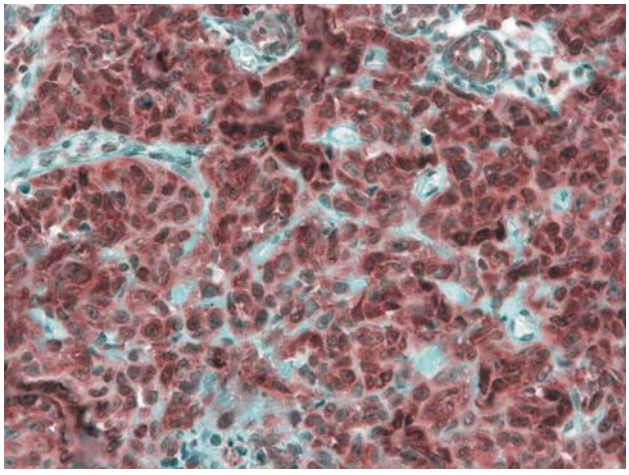

Figure 3. *Additional cytoplasmic staining of MAD2 in a MAD2-over-expressed oral squamous cell carcinoma, original magnification, ×400).*

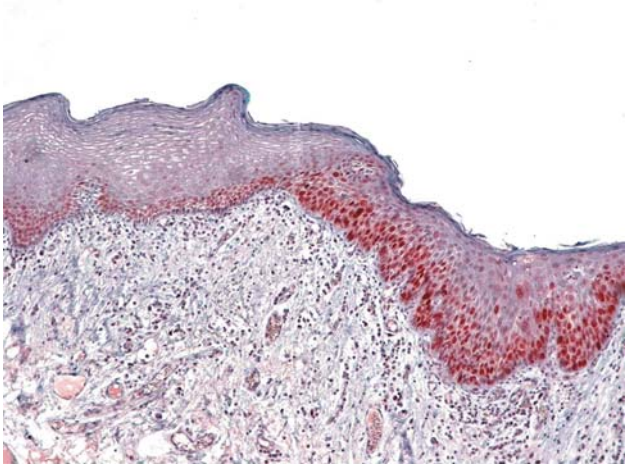

Figure 4. *MAD2 expression in dysplastic (right) compared to normal (left) oral epithelium: MAD2-positive cells are more numerous and located also above the parabasal layer, original magnification, ×200).*

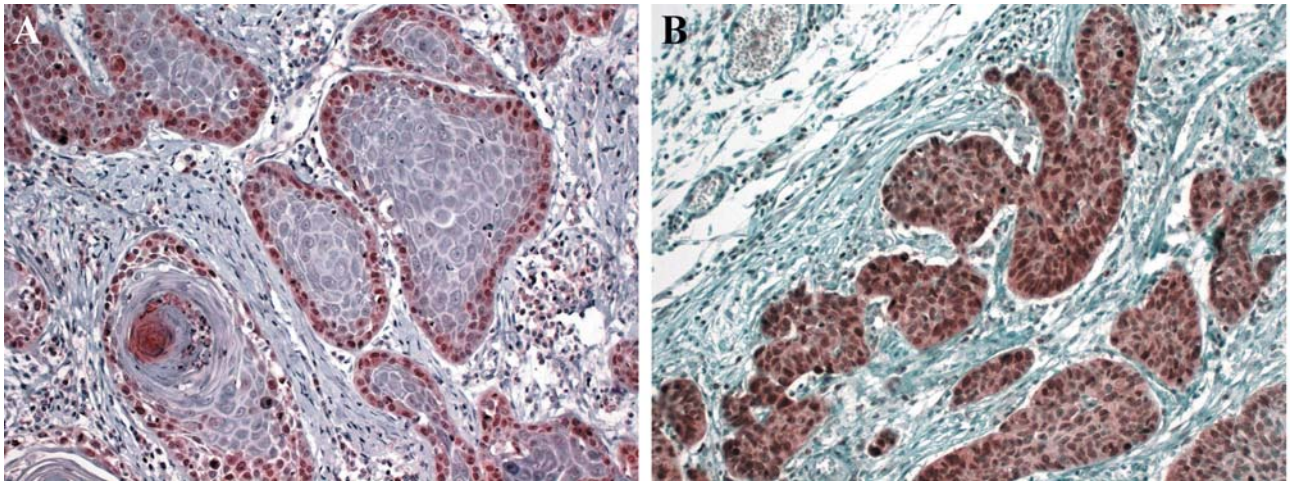

Figure 5. *Different patterns of MAD2 expression in a well- (A) and in a less- differentiated (B) oral squamous cell carcinomag, original magnification, ×200).*

et al. reported less nuclear and relatively increased cytoplasmatic MAD2 levels as an explanation for an impaired spindle checkpoint function in testicular germ cell tumors (12). Such observation has been confirmed by the immunohistochemical analysis of Burum-Auensen *et al*. (13). Another report supporting these findings is the in vitro study by Kasai and collaborators on MAD2 in human T-cell leukemia virus Type I transformed cells that revealed a correlation between the dislocation of MAD2 from the nucleus to the cytoplasm and the loss of spindle checkpoint function in these cells (36). Such a loss of function may be related to that MAD2, localized in the cytoplasm displaced from its binding motifs in the nucleus, prevents it to fulfil its role as a spindle checkpoint mediator (13). Even though we observed this phenomenon in oral squamous cell carcinomas with MAD2 over-expression, we have not been able to demonstrate in such tumors a spindle checkpoint dysfunction in the form of an abnormal DNA content. Since not all cancer cells with aneuploidy had an impaired spindle checkpoint, another possible explanation is that cancer cell aneuploidy may arise from alternative defects yet to be discovered (3).

Table I. *Correlation between MAD2 expression and clinicopathological parameters.*

Parameters	MAD2 expression (No. of cases)		*p*-Value
	Low	High	
Histologic grade			<0.001
G1	12	0	
G2	14	6	
G3	5	12	
TNM classification			
pT			1
pT1	9	7	
pT2	14	7	
pT3	4	3	
pT4	4	1	
pN			0.034
pNx	7		
pN0	10	5	
pN1	9	1	
pN2	7	10	
Stage			0.146
X	7		
I	2	1	
II	7	1	
III	8	3	
IVa	9	11	
Regional metastasis			0.746
X			
Absent	10	5	
Present	16	11	
Recurrence			0.754
Absent	20	13	
Present	11	5	
Ki-67 labeling index			<0.001
Low	28	3	
High	3	15	
DNA content			0.190
X	11		
Diploid	16	7	
Aneuploid	7	8	
BUBR1 expression			0.286
No over-expression	26	12	
Over-expression	5	6	

Finally, we have shown that the ratio of MAD2 expression in neoplastic tissues to that in its normal mucosa tissues (C/N ratio) more than 2 was not only statistically significantly correlated to Ki-67 labeling index (p=0.007) but also had a relationship with histological grade, although without statistical significance (p=0.1). A C/N ratio of MAD2 expression greater than 2 has been previously related to a more advanced histologic grade in gastric carcinoma (4) and to a greater frequency of metastases not only in gastric carcinoma (4, 15) but also in colorectal carcinoma (5). These findings suggest that a high C/N ratio of MAD2 expression may be clinically an important indicator of worse prognosis in human carcinomas. In our study, the C/N ratio of MAD2 expression had a tendency to recapitulate MAD2 expression, given its relationship with the grade of differentiation of the tumor and its proliferative activity. Since the difference in the protein level between cancer and normal mucosa is easily detected by immunohistochemistry, MAD2 protein might also be a good marker of poor prognosis in squamous cell carcinoma of the oral cavity.

In conclusion, these findings, as a whole, lead us to speculate that MAD2 over-expression is likely to represent an important transformation factor associated with a more malignant phenotype of squamous cell carcinoma of the oral cavity. MAD2 testing might have a clinical role in predicting prognosis, selecting appropriate chemotherapeutic protocols and providing novel strategies for oral cancer therapy.

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