Specific induction of the high-molecular-weight microtubule-associated protein 2 (hmw-MAP2) by betel quid extract in cultured oral keratinocytes: clinical implications in betel quid-associated oral squamous cell carcinoma (OSCC)

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Betel quid (BQ) chewing, a popular habit in numerous Asian countries including India and Taiwan, has a strong correlation with an increased risk of oral squamous cell carcinoma (OSCC). While substantial efforts have been made to test the cytotoxic, genotoxic and mutagenic effects of BQ extract and its components, the disease mechanisms underlying BQ-induced oral carcinogenesis remain obscure. Here, we show that a neuronal protein, microtubule-associated protein 2 (MAP2), was induced by BQ extract in cultured normal human oral keratinocytes (NHOKs). Subsequent analyses demonstrated that such induction was more eminent and consistent in the high-molecular-weight isoform of MAP2 (hmw-MAP2) than that in its low-molecular-weight counterpart (lmw-MAP2). Furthermore, we analyzed expression of the hmw-MAP2 protein in 88 oral specimens consisting of clinicopathologically pre-malignant (leukoplakia) and malignant (OSCC) lesions, along with their adjacent normal mucosa. Immunohistochemistry revealed that, with the exposure to BQ, the hmw-MAP2 was over-expressed in 41.2% (7/17) of OSCC, 11.2% (1/9) of BQ-free OSCC. These results suggest a significant correlation between expression of the hmw-MAP2 and BQ-associated progression of oral carcinogenesis (P = 0.0046). Interestingly, the hmw-MAP2 was found to preferentially express in histopathologically less differentiated OSCC (P = 0.014); the percentages of positive staining in poorly, moderately and well differentiated OSCC were 62.5, 21.4 and 7.1%, respectively. However, BQ chewing appeared to have marginal correlation with such propensity. Finally, we show that the majority of hmw-MAP2-positive poorly differentiated lesions were also histopathologically invasive. Taken together, these findings suggest the possibility that the hmw-MAP2 may be a diagnostic marker for BQ-chewing lesions and a potential therapeutic target. To our knowledge, this study has provided the first clinical implication that closely links a cytoskeletal protein to BQ-associated oral cancer.

Introduction

Betel quid (BQ) chewing is a prevalent habit in India and many Southeast Asian countries including Taiwan, in which an estimate of 2 million BQ chewers is constantly reported (1,2). Epidemiological evidence has firmly established a causal relationship between BQ chewing and high incidence of oral cancer (3). Indeed, in Taiwan, oral squamous cell carcinoma (OSCC) accounts for the fourth leading cause of cancer mortality and the fifth most common cancer in males (4), while, astoundingly, ~80% of oral cancer deaths are associated with BQ chewing (5). Hence, BQ chewing is generally believed to be the major etiological factor of oral cancer. Recently, BQ-related etiology and the underlying disease mechanisms has been an emerging subject intensively studied, despite that most of the reports are focused on cytotoxicity, genotoxicity and mutagenicity of BQ extract and its individual ingredients. For example, it has been shown that betel nut extract and arecoline, one of the betel nut alkaloids, suppress the growth of cultured normal human oral keratinocytes (HNOKs) and induce DNA damage in a dose-dependent manner (6–8). Induction of reactive oxygen species during BQ chewing has been demonstrated to be involved in tumor initiation by inducing genotoxicity and mutagenicity (9–11). In addition, previous studies reveal that betel nut extract induces neoplastic lesions in experimental animals (12,13). Overall, these findings strongly imply that BQ is a chemical carcinogen closely associated with oral carcinogenesis. However, the precise mechanisms, from molecular and biochemical standpoint, by which BQ induces OSCC have not been well studied.

It is a widely accepted notion that tyrosine kinases play central roles in the growth and differentiation of normal and tumor cells. This is supported by the fact that tyrosine kinases are engaged in a plethora of signal transduction pathways conveying growth cues and therefore are more susceptible to be aberrantly activated as oncogenes. Thus, to gain better understanding of BQ-induced oral carcinogenesis, we chose to selectively study tyrosine kinase expression profile of primary keratinocytes derived from normal human oral mucosa in the presence of BQ treatment. Surprisingly, we identified, presumably due to sequence similarity, a non-kinase candidate known as microtubule-associated protein 2 (MAP2) that is up-regulated in response to BQ treatment. Expression of MAP2 is largely confined to the nervous system being one of the most abundant proteins in brain. However, despite being relatively rare, MAP2 can also be expressed in some non-neuronal tissues such as testis and muscle (14,15). Multiple human MAP2 isoforms resulting from alternative splicing of a primary transcript have been classified into two groups: (i) high-molecular-weight MAP2 (hmw-MAP2), which includes MAP2A and MAP2B, with molecular weights of ~280 kDa; and (ii) low-molecular-weight MAP2 (lmw-MAP2), which includes MAP2C and MAP2D, with molecular weights close to 70 kDa. We then examined induction of MAP2 at both RNA and
protein levels in cultured NHOKs, followed by investigating expression of the hnm-WAP2 in human oral tissue specimens including normal mucosa, leukoplakia and OSCC that are clinicopathologically corresponding to normal, pre-malignant and malignant, respectively, with or without BQ exposure. The implications of our study in the disease mechanisms and clinical applications of BQ-associated oral carcinogenesis will also be discussed.

Materials and methods

Tissue preparation

Surgical resection specimens containing 18 leukoplakia, 36 squamous cell carcinoma and 34 adjacent normal mucosa were obtained from patients undergoing maxillofacial surgery. Afterward, the tissues were immediately embedded in OCT compound and frozen at −70 °C. These tissues were used in immunohistochemistry. For primary culture, fresh oral mucosa were obtained during the surgery and stored at 4 °C in culture medium. IRB of National Taiwan University Hospital approved the use of these tissues.

Primary culture of NHOKs

Human oral mucosa were rinsed twice with Ca2+ and Mg2+-free HBSS (Sigma) and cut into 1 × 1 × 1 mm3 small pieces. Subsequently, the explants were digested in HBSS containing 0.6 U/ml of Dispase (Gibco, Invitrogen) and 100 U/ml of collagenase (Sigma) at 37 °C for 1 h. The resulting cell mass after digestion was then washed in HBSS four times and then pelleted at 100 g for 5 min. After resuspension in the culture medium Defined Keratinocyte-FMEM (Gibco, Invitrogen) supplemented with bovine pituitary extract (Upstate Biotechnology, Lake Placid, NY) at 50 μg/ml, the cells were then seeded onto 6-well culture plates and incubated at 37 °C in a humidified 5% CO2 incubator. Cells grown for 10–14 days at ~70% confluency were used in this study.

BQ extract preparation

110 g of BQ were purchased from a local vendor in Taipei city. BQ used in this study consists of betel nut, inflorescence of Piper betle Linn. and lime. BQ extract preparation was similar to that of areca nut extract as described by Jeng et al. (16) with minor modifications. Briefly, BQ quid was smashed into thick paste in a blender and the ingredients were then extracted by 250 ml of ddH2O at 4 °C overnight with constant stirring. After crude sifting through cheesecloth to remove insoluble BQ debris, the extract was spun at 10,000 g for 15 min. The supernatant was then subjected to further filtration by 0.45 μm filters to eliminate residual insoluble precipitates prior to lyophilization. Powdered extracts were redissolved in culture medium, sterilized by 0.22 μm filters and stored at −70 °C with working aliquots.

Kinase display and identification of MAP2

Kinase display was performed as described by Robinson et al. (17,18) with minor adjustments. Total RNA was isolated by TRIZOL Reagent (Life Technologies) of which 25 μg was reverse-transcribed using oligo(dT)-priming ThermoScript RT–PCR System (Life Technologies). The cDNA was purified by CHROMA SPIN-200 columns (Clontech, Palo Alto, CA) and used as the template in subsequent PCR reactions. Degenerate PCR primers are derived from the conserved motifs DFG and DYW of known kinases, with an asymmetric BstEII site incorporated at the 5′ end of each primer. For primer labeling, 1.25 μl of 5′ primers (10 μm each), 1 μl of 5′+3′ polynucleotide kinase buffer (New England Biolabs, Beverly, MA), 2.5 μl of [γ-33P] ATP (10 μCi/μl) and 0.25 μl of T4 polynucleotide kinase (Biolabs) were assembled into a final 5 μl reaction and incubated at 37 °C for 30 min. Afterward, the enzyme was inactivated at 65 °C for 5–7 min. The thermal cycling features including a two-step amplification are as follows: initial four cycles of 93 °C for 45 s, 44 °C for 1 min 30 s and 72 °C for 12 s, then 28 cycles of 93 °C for 45 s, 56 °C for 1 min 30 s and 72 °C for 15 s. The PCR products were run on a 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) and a 170 bp fragment was gel-purified by QIAEX Agarose Gel Extraction Kit (Qiagen). Purified PCR products (10 μl c.p.m/μl) were equally divided into 15 aliquots and digested with 15 restriction enzymes separately (AciI, AatII, BosI, Bsp1286, BstNI, HaeIII, HinfI, HpaII, MboII, Rsal, SstII, Hhal, NciI and AvalII) at 37 or 60 °C for 1 h. After stopping the reactions at 65 °C for 7 min, the digests were resolved in 7% polyacrylamide/urea gel. Gels were then dried for 50 min, exposed for 2 h and developed by autoradiography. To identify the novel MboII-digested cDNA fragment, the corresponding 48 bp band was excised from the gel and DNA extracted by boiling in 100 μl ddH2O for 5 min. The cDNAs were then end-to-end (3′ to 3′) rejoined by sticky- or blunt-end ligation and cloned into the pGEM-T Easy Vector (Promega). Bacterial transformation was conducted by electroporation using ElectrosMax DH10 B competent cells (Life Technologies) and positive clones were identified by blue/white selection. The end sequences were determined and used to BLAST search TIGR Unique Gene Indices.

Semi-quantitative RT–PCR

Five micrograms of total RNA was converted into cDNA as described above in a volume of 20 μl. Ten-fold cDNA dilutions were used as templates and amplified by Amplitaq Gold polymerase (Applied Biosystems) and MAP2-specific primers as follows: P (all variants): 5′-CAAATGTGGCTCTCTCAGAGACAAACA-3′ (forward) and 5′-ATGATGGATAGCTCTCGACCA-3′ (reverse); P (V1 + V3): 5′-ATCTCCCTCAGGTAAGCGAG-3′ (forward) and 5′-GCAGTGGGTGAATTAAGC-3′ (reverse); and P (V2 + V4): 5′-TGCCCTAGACAGCAGTCCACA-3′ (forward) and 5′-GCGAGTGGTGGAATTAAAAGC-3′ (reverse). PCR conditions comprised 94 °C for 12 min followed by 50 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, then a final 72 °C for 7 min.

Western blot analysis

BQ-treated (1600 μg/ml) NHOKs were washed twice with PBS and lysed in RIPA buffer [100 mM Tris (pH 8.2), 150 mM NaCl, 1% deoxycadic acid, 1% Triton X-100, 0.1% SDS and 5 mM EDTA] supplemented with protease inhibitor cocktail tablets (Roche, Mannheim, Germany). Protein concentrations of cell extracts were determined by BCA protein assay reagent (Pierce) using BSA as standard. Total cell extracts (12 μg/lane) were resolved on 3–8% Tris–acetate gels (Invitrogen) followed by transfer to Immobilon-P PVDF membrane (Millipore). Primary antibody incubation was performed with mouse anti-MAP2 antibody (Zymed Laboratories, South San Francisco, CA) at 1:1000 or rabbit anti-MAP2 antibody (Chemicon, Temecula, CA) at 1:2000 dilutions at room temperature for 1–2 h. Secondary antibody incubation was done with anti-mouse or anti-rabbit IgG-HRP (Amersham Pharmacia) at 1:10000 dilutions at room temperature for 1 h. Detection of the blot was done with ECL chemiluminescence kit (PerkinElmer Life Sciences).

Immunohistochemistry

Immunohistochemical staining of 5-μm-thick frozen sections was conducted using Streptavidin–Biotin Universal Detection System (Immunotech, LOCATION?). Incubation of primary antibody was mouse anti-MAP2 antibody (Zymed Laboratories, LOCATION) at 1:100 dilution or mouse anti-cytokeratin (Pan) (Zymed Laboratories) at 1:150 dilution for 1 h. Interpretations of staining results were performed by a pathologist from the Department of Pathology at National Taiwan University Hospital.

Results

Effects of BQ extract on the growth of cultured NHOKs

to determine the optimal concentration of BQ extract for treating cultured NHOKs, BQ extract at concentrations of 500, 800 or 1600 μg/ml were incubated with the cells throughout a 5-day period. Cytotoxicity was evaluated by measuring lactate dehydrogenase activity released from the cytosol of damaged cells into the culture medium at days 1, 3 and 5, respectively, following exposure to BQ extract. We decided to use the concentration at 1600 μg/ml for subsequent kinase display because it not only best resembles the concentration detected in saliva during BQ chewing (19), but also caused ~5–15% additional cell death that meets our preferred cytotoxicity considering limited experimental materials generally obtained from primary cultures (Figure 1A). Morphologically, untreated cultured NHOKs feature cuboidal or polygonal in appearance and sharply defined intercellular spaces. Exposure of the cells to BQ extract at 1600 μg/ml resulted in marked cell retraction, increasing formation of cytoplasmic vacuoles and diminishing intercellular spaces, as the incubation proceeded along the 5-day period (Figure 1B).

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Identification of MAP2 induction in BQ-treated cultured NHOKs

To identify differential expression of tyrosine kinase(s) in response to BQ treatment, we performed kinase display using RNA obtained from mock- and BQ-treated (1600 µg/ml) cultured NHOKs. A pool of degenerate primer pairs, which is derived from the conserved motifs of virtually all known tyrosine kinases, amplified a 48 bp MwoI-digested cDNA fragment exhibiting increasing expression in response to BQ treatment on the polyacrylamide gel. Unlike other MwoI-digested cDNAs, this particular cDNA fragment was unable to correlate a known tyrosine kinase of any category in our database (Figure 1C). We therefore gel-purified and cloned this cDNA fragment. Sequence analysis, by BLAST search against TIGR Gene Indices, revealed that the cDNA fragment matched partially to a sequence residing within exon 7 (Figure 2A) of microtubule-associated protein 2 (MAP2; THC1234859). Surprisingly, these clones identified only the high-molecular-weight variants of MAP2 (hmw-MAP2), owing to the lack of exon 7 in the low-molecular-weight variants (lmw-MAP2), which, in company with the hmw-MAP2, are alternatively spliced isoforms of the canonical MAP2 transcript (Figure 2A). To confirm up-regulation of the hmw-MAP2 consisting of variants 1 and 3 (MAP2B and MAP2A, respectively) as illustrated in Figure 2A, a set of nested primers, primer V1 + V3, was designed to specifically amplify the hmw-MAP2 in semi-quantitative RT–PCR reactions, using the same batch of template cDNA as in kinase display. Importantly, induction of hmw-MAP2 mRNA was evident throughout the BQ incubation period (Figure 2B), consistent with the result of kinase display. Since we surmised that BQ may also affect expression of the lmw-MAP2 including variants 2 and 4 (MAP2C and MAP2D, respectively), we thus conducted the same experiment except for the primer set being used; primer V2 + V4 amplified a fragment (510 bp) representing the lmw-MAP2, which was sufficiently separated from that of the hmw-MAP2 (4.5 kb; data not shown). Interestingly, lmw-MAP2 mRNA was also found to be induced by BQ, despite its eschew from being identified initially by kinase display (Figure 2B).

BQ induces protein expression of the hmw-MAP2 in cultured NHOKs

We next asked whether MAP2 is induced by BQ at protein level. Western blotting showed that the hmw-MAP2 was induced by BQ extract in cultured NHOKs derived from three independent patients, with induction maximizing at 48 h after BQ treatment (Figure 3A and B). Densitometry analysis, as shown in Figure 3B, further revealed that such peak induction levels in patient #1, #2 and #3 were 2.87-, 1.8- and 2.42-fold, respectively, relative to the mock-treated control. The lmw-MAP2, in contrast, exhibited marginal induction by BQ extract and remained relatively steady induction levels throughout the incubation period, reminiscent of its mRNA induction pattern (Figure 3A and B). At this point, we decided to focus on the hmw-MAP2, as it appears more adherently associated with BQ induction without being susceptible to individual variations and therefore could potentially play a...
critical role in BQ-associated clinicopathological diseases such as OSCC. In addition, at day 2 of BQ extract incubation, the hmw-MAP2 was clearly localized in the cytoplasm of cultured NHOKs, similar to its localization in neuronal cells. Notably, over-expression of the hmw-MAP2 resulting from BQ induction did not lead to aberrant cellular localization, as identical localization pattern was observed, despite a lesser degree of staining intensity, in untreated cells (data not shown).

Expression of the hmw-MAP2 correlates with the progression of oral carcinogenesis in a BQ-specific fashion
To test whether the hmw-MAP2 is specifically over-expressed in BQ-exposed OSCC, oral tissue sections containing normal mucosa, leukoplakia or tumor lesions of distinct differentiation states were subjected to immunohistochemical staining using anti-hmw-MAP2 antibody. In the presence of BQ exposure, expression of the hmw-MAP2 correlated with the progression of oral carcinogenesis ($P = 0.0046$), culminating in its over-expression (or positive staining) in 41.2% of OSCC while none or only 11.2% were found in normal mucosa or leukoplakia, respectively (Table I). In contrast, in the absence of BQ exposure, of a total of 19 OSCCs tested, only two or 10.5% of which scored positive, resulting in a lack of correlation between expression of the hmw-MAP2 and development of OSCC ($P = 0.421$) (Table I). A representative case shown in Figure 4 demonstrates that BQ-induced over-expression of the hmw-MAP2 coincides with the development of OSCC; virtually no hmw-MAP2 staining in normal mucosa, weak to moderate staining predominantly in basement membrane of leukoplakia and strong intensity, an estimate of >80% of tumor cells, in OSCC. Thus, these findings suggest that specific induction of the hmw-MAP2 in human oral squamous cells is likely to play a role in the BQ-associated tumorigenic process.

Preferential over-expression of the hmw-MAP2 in less differentiated yet invasive OSCC
In the course of histopathological examination of the OSCC under study, we surprisingly found that 62.5% (5/8) of poorly differentiated OSCC (PD-OSCC) displayed positive staining of the hmw-MAP2, irrespective of BQ exposure. Unlike BQ-free PD-OSCC, in which merely two out of five scored positive, all three BQ-exposed PD-OSCCs were moderately to strongly stained by anti-hmw-MAP2 antibody (Table IIA). Noticeably, three moderately differentiated OSCCs (MD-OSCC) and one well differentiated OSCC (WD-OSCC) were also considered over-expression of the hmw-MAP2 in the presence of BQ, whereas expression was essentially undetectable in their counterparts devoid of BQ exposure. A typical example is shown in Figure 5, in which the hmw-MAP2 is highly expressed in PD-OSCC, in sharp contrast with that in WD-OSCC. It is suggested that histopathological morphology
of invasive SCC features small cords or strands of infiltrating tumor cells disseminating into submucosa (Figure 5), and that less differentiated tumors display more tendency to be aggressive and invasive. Indeed, six out of a total of eight PD-OSCCs were diagnosed as invasive OSCC and interestingly, of which four (67%) have staining equal or greater than moderate intensity of the hmw-MAP2, regardless of the presence or absence of BQ exposure (Table IIB). Thus, we conclude that the hmw-MAP2 is preferentially over-expressed in less differentiated (P \leq 0.014) yet invasive OSCC, despite minor correlations between this tendency and BQ-chewing habit (P = 0.054 and 0.044 for BQ-chewing and BQ-free OSCCs, respectively).

### Discussion

We have exploited primary culture of oral keratinocytes and kinase display aiming to unravel the long-standing mystery as to how mechanistically BQ chewing leads to oral cancer in the early stage. After semi-quantitative RT-PCR confirmation on several differentially expressed genes detected by kinase display, two tyrosine kinases, FRK and BRAF, were found to be up-regulated in response to BQ treatment in cultured NHOKs. In addition to identifying these kinases, we fortuitously identified a highly induced non-kinase candidate, microtubule-associated protein 2 (MAP2). We argue that the serendipity was due to sequence similarity between the degenerate primers and partial cDNA sequences of MAP2, as sequence alignment revealed that a stretch of 17mer oligonucleotides harbored by the 5' primers exactly matched a counterpart in exon 7 of primary MAP2 transcript. Subsequent structural analysis indicated that exon 7 is absent from the lmw-MAP2, thereby verifying that the hmw-MAP2 was the exclusive MAP2 isoform identified initially by kinase display. Significantly, however, induction of the hmw-MAP2 was confirmed at both RNA and protein levels.

In the adult brain, the hmw-MAP2 is predominantly located in dendrites and is virtually absent from axons (20,22). Elevated expression of the hmw-MAP2 in dendrites has been implicated in facilitating neurite outgrowth and establishing neuronal polarity and plasticity (23–25). Moreover, previous studies show that outgrowth of cytoplasmic elongations becomes evident when MAP2 is over-expressed in cultured

![Fig. 3. Induction of MAP2 protein in vitro in response to BQ extract.](image-url)
non-neuronal cells (26–28). Accordingly, our observations that BQ extract induces protein over-expression of the hmw-MAP2 by as high as 2.87-fold and that the over-expressed hmw-MAP2 protein is cytoplasmically localized (data not shown) may thus account for the resultant morphological changes of NHOKs in response to BQ treatment. Phosphorylation is thought to be the most prevailing post-translational modification in microtubule-associated proteins (MAPs) by which their cellular functions are primarily modulated. In support of this view are the results that MAPs, including MAP2, are highly phosphorylated in vivo by signaling pathways that control neuronal polarity and plasticity (29–32). Nonetheless, it remains to be seen whether BQ-induced over-expression of the hmw-MAP2 and the observed morphological changes of NHOKs is mediated by similar signaling pathways.

Previous implications of MAP2 involvement in tumorigenic process have been relatively limited, yet our findings provide the first indication of MAP2 in oral cancer. Recent studies indicate that MAP2 is over-expressed in cutaneous melanoma (33) and is a specific marker for pulmonary carcinoid tumor and small cell carcinoma (34). Leukoplakia are the most common pre-malignant or pre-cancerous lesions of OSCC (35,36). Therefore, our immunohistochemical staining results of normal mucosa, leukoplakia, and OSCC suggest that expression of the hmw-MAP2 significantly correlates with progression of BQ-induced OSCC ($P \leq 0.0046$), since the percentage of hmw-MAP2-positive staining is as much as 4-fold in BQ-exposed OSCC (41.2%) as in BQ-free OSCC (10.5%), whereas normal mucosa and leukoplakia show much less immunoreactivity as a whole. However, two of the BQ-free OSCCs exhibit strong staining intensity, which we conjecture is due to individual variations presumably resulting from unidentified etiological parameters. Furthermore, the hmw-MAP2 is shown to be preferentially expressed in histopathologically less differentiated OSCC ($P \leq 0.014$). This is surprising in light of the fact that MAP2 has been used extensively as a specific marker for neuronal differentiation in the past two decades (37,38). Given that carcinogenesis is a multi-step

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**Table II.** The hmw-MAP2 expression in the histopathologically distinct OSCCs with or without BQ chewing habit

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<tr>
<th></th>
<th>No BQ-chewing</th>
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<th>Total OSCC</th>
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<td></td>
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<tr>
<td>WD-OSCC</td>
<td>8</td>
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<td>5</td>
<td>1 (16.7%)</td>
<td>13</td>
<td>1 (7.14%)</td>
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<td>5</td>
<td>3 (37.5%)</td>
<td>11</td>
<td>3 (21.4%)</td>
</tr>
<tr>
<td>PD-OSCC</td>
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<td>3 (100%)</td>
<td>3</td>
<td>5 (62.5%)</td>
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<td>17</td>
<td></td>
<td>36</td>
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B

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<tr>
<th></th>
<th>Invasive OSCC</th>
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<tr>
<td></td>
<td>2 (50%)</td>
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<td>0</td>
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<td>4 (67%)</td>
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$^a$The correlation between staining of the hmw-MAP2 and differentiation status of OSCC was analyzed by $\chi^2$ test.
process wherein cells undergo a spectrum of differentiation defects that result in loss of proliferative control (39–41), it requires further investigation to illuminate such a paradoxical scenario, i.e. potentially ‘dedifferentiated’ role of MAP2 during late stage of tumor development. Finally, we show that the hmw-MAP2 is highly expressed in the majority of invasive OSCC (67%). Consequently, this finding, in conjunction with the previous one, raises a possibility that over-expression of the hmw-MAP2 may play a critical role in the development of highly malignant OSCC characteristic of invasive and/or metastatic.

Although our data suggest a strong clinical correlation between expression of the hmw-MAP2- and BQ-associated oral carcinogenesis, a major question immediately ensues, namely how mechanistically the hmw-MAP2 is involved in the transition from normal mucosa to OSCC? As noted above, MAP2 has been shown to be a substrate for many protein kinases defining a multitude of signal transduction pathways, and, more importantly, a number of these protein kinases have been implicated in oncogenic transformation (42–46). Thus, it is probable that BQ activates MAP2-tethered signal transduction pathways and that persistent exposure of BQ results in constitutive activation of the pathways, which in turn leads to neoplastic growth. In this context, the hmw-MAP2 is anticipated to play an indirect role in BQ-induced oral carcinogenesis; perhaps over-expression of the hmw-MAP2 is a second effect of these pathways. Further studies pertaining to the issue are underway. Suffice it to say that the identification of specific induction of MAP2 in BQ-associated OSCC has opened up a new avenue for dissecting the detailed disease mechanism. In conclusion, we propose that the hmw-MAP2 could be an early diagnostic marker for BQ-induced oral cancer especially based upon its specific induction by BQ and its emergence in some cases of leukoplakia, a pre-cancerous state of OSCC. Likewise, the hmw-MAP2 may also be a specific indication for less differentiated and invasive lesions, according to our immunohistochemical results. Since taxol, a microtubule stabilizer, has been tentatively used for anticancer therapy (47,48), our findings thus raise an intriguing possibility that agents functionally antagonistic to taxol may be valuable for therapeutics of BQ-associated oral cancer, in view of the fact that MAP2 has been shown to stabilize and stiffen microtubules (49–51).

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References
