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Involvement of microtubule-associated protein 2 (MAP2) in oral cancer cell motility: A novel biological function of MAP2 in non-neuronal cells

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Abstract

Microtubule-associated protein 2 (MAP2) has been better known for its well-defined role primarily in neurite outgrowth during neuronal development. However, the biological functions of MAP2 in non-neuronal cells, such as epithelial cells, remain largely unknown. In the present study, we sought to investigate the cellular functions of MAP2 by separately establishing stable expression of two MAP2 isoforms, MAP2A and MAP2C, in oral squamous cell carcinoma, Ca9-22. Ectopic expression of MAP2A or MAP2C results in microtubule bundling predominantly at the cell periphery. Remarkably, overexpression of MAP2A but not MAP2C significantly promotes migration of Ca9-22 cells, whereas knockdown of MAP2A expression by specific siRNA oligos dramatically decreases cell migration of HaCaT, an immortalized keratinocyte cell line with abundant endogenous MAP2A. Furthermore, by immunohistochemical studies, MAP2A was shown to highly and selectively express in invasive oral cancer tissues, consistent with its motility-promoting cellular function revealed through *in vitro* assays. Thus, our findings have not only identified a novel role of MAP2 in non-neuronal cells, but also provided the first implication of MAP2 in malignant oral cancer tissues.

Keywords: Microtubule-associated protein 2 (MAP2); Oral cancer cells; Microtubule bundling; Cell migration; Invasive oral cancer tissues

Microtubule organization in the cytoplasm is largely modulated by microtubule-associated proteins (MAPs), among which microtubule-associated protein 2 (MAP2) is a neuron-specific protein relatively abundant in the mammalian central nervous system [1–4]. Human MAP2 contains MAP2A and MAP2B, the long isoforms, and MAP2C and MAP2D, the short isoforms, due to alternative splicing of a single transcript. MAP2 is best known for its importance in neuronal morphogenesis. This is mainly revealed by its essential role in neurite outgrowth [5,6]. No neurite growth is observed when MAP2 expression is suppressed by specific antisense oligonucleotides in neuronal cell cultures [7]. In contrast, neurite-like protrusions can be induced in non-neuronal cells by exogenous overexpression of MAP2 [8]. It is generally believed that MAP2 potentiates neurite outgrowth through rearranging cytoskeletal organization especially microtubule dynamics. In addition, the function of MAP2 is regulated

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predominantly dependent upon phosphorylation [9]. Of particular relevance in this regard are the observations that MAP2 proteins are highly phosphorylated *in vivo* and are excellent substrates for many protein kinases and phosphatases *in vitro* [10]. It is likely that phosphorylation of MAP2 may influence its interaction with microtubules and thus cell morphology and plasticity.

Microtubule network has been suggested to engage in regulating cell migration. This is supported by the evidence that destruction of microtubules in fibroblasts results in inhibition of lamellipodial protrusion, a cellular structure formed at the front of migrating cells [11]. Moreover, recent studies show that microtubule dynamics is critical for migration of neuronal and endothelial cells [12–15]. Indeed, MAP2, the major regulator of microtubule dynamics, has been shown to functionally participate in both neurite outgrowth and neuronal migration. For instance, MAP2-knockout mice exhibit retarded neuronal migration [16], indicating the requirement of MAP2 in neuronal cell motility. Given that non-neuronal cells acquire neurite-like cell protrusions when MAP2 is ectopically expressed [8], it is plausible that MAP2 may function in regulating migration of non-neuronal cells such as epithelial cells.

We have previously shown that MAP2 is specifically induced by betel quid (BQ) extract in cultured normal human oral keratinocytes (NHOKs). Moreover, our data indicate that MAP2 is highly expressed in BQ-chewing oral squamous cell carcinoma (OSCC) [17]. Here, we sought to define a novel biological role for MAP2 by demonstrating its cellular function in oral cancer cells of epithelial origin. We found that ectopic expression of MAP2A enhanced cell migration, while knockdown of endogenous MAP2A by siRNA reduced cell migration. On the other hand, we showed that MAP2A was highly and selectively expressed in clinicopathologically invasive oral cancer tissues but not in normal mucosa. These results have suggested a possible biological function for MAP2 in oral cancer cell metastasis.

Materials and methods

Plasmid constructions. Full-length MAP2A cDNA was amplified using pSV-MAP2A plasmid as the template and the PCR primers were: 5′-TCCCGGGATCCAATGGCCGACGAGCGGAAA-3′ and 5′-GAATAGGGCCCTCACAAGCCCTGCTTAGCAAG-3′. The PCR product was digested with BamHI and XhoI, cloned into the BamHI/XhoI site of pCDNA 3.0-HA expression vector, and then sequenced (pCDNA3.0-HA-MAP2A). For construction of pCDNA3.0-HA-MAP2C plasmid, full-length MAP2C cDNA was amplified using pRc/CMV-MAP2C as the template and the primer sequences were: 5′-TCCCGGGATCCAATGGCAGATGAACGGAAA-3′ and 5′-ACATGCTCGAGTCACAAGCCCTGCTTAGCGAG-3′. The cDNA fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced, and then subcloned into BamHI/XhoI site of pCDNA3.0-HA. Construction of EGFP-MAP2C plasmid was done by direct cloning of the BamHI/XhoI fragment from pCDNA3.0-HA-MAP2C into BglII/SalI site of pEGFP-C3 vector. EGFP-MAP2A was constructed by first subcloning the SalI/AseI fragment of pSV-MAP2A into SalI/NdeI site of pT7Blue vector (Novagen). Subsequently MAP2A was cloned into pEGFP-C3 by a two-step ligation of N-terminal with C-terminal fragments. The N-terminal fragment was generated by PCR amplification of the template pT7Blue-MAP2A using primer sequences: 5′-TCCCGGGATCCAATGGCCGACGAGCGGAAA-3′ and 5′-TCTTGGGATATCCTCTAG-3′. This PCR fragment was cut with BamHI and KpnI and cloned into the BglII/KpnI site of pEGFP-C3 (EGFP-N-terminal-MAP2A). The C-terminal fragment was generated by cutting pT7Blue-MAP2A with KpnI and BamHI and then cloned into KpnI/BamHI site of EGFP-N-terminal-MAP2A, completing EGFP-MAP2A construction.

Cell culture, plasmid DNA transfection, and stable transfectant constructions. Oral cancer cells, Ca9-22, were maintained in Dulbecco's modified Eagle medium supplemented with nutrient mixture F-12 (DMEM/F12) (Invitrogen Technologies, Carlsbad, CA) and 10% FBS. All plasmids constructed above were introduced into Ca9-22 cells by using FuGENE 6 transfection reagent (Roche Applied Science). After transfection (36–48 h), cells were harvested for subsequent experiments. For construction of stable transfectants, Ca9-22 cells were transfected with pCDNA3.0-HA-MAP2A or pCDNA3.0-HA-MAP2C, and stable transfectants were selected by G418 (1.5 mg/ml) for 2 months. Positive clones with stable expression of MAP2A or MAP2C were verified by Western analysis.

Immunoblotting. Immunoblotting was performed as previously described [17]. Primary antibodies were mouse anti-HA (Covance, Berkeley, CA) and mouse (Zymed) or rabbit anti-MAP2 (Chemicon).

Immunofluorescence. Ca9-22 cells (5×10^4) were seeded on coverslips in 24-well dishes. Cells were then fixed with cold methanol at −20 °C for 20 min followed by blocking in PBS containing 3% BSA at RT for 30 min. Primary antibody incubation was carried out at RT for 1 h using anti-HA at 1:200 dilution or anti-β-tubulin (Chemicon) at 1:100 dilution. After washing, the cells were incubated with both FITC-conjugated anti-mouse IgG and Texas-Red conjugated anti-rabbit IgG (Jackson Laboratories) for 1 h, and with DAPI (Roche) during the last 10 min of this 1-h incubation. Cells were mounted with anti-fading medium (Dako Cytomation) and visualized under fluorescence microscope.

Immunohistochemistry. Surgical resection specimens including normal and cancer tissues were obtained from patients undergoing maxillofacial surgery. Invasive tumor tissues were histopathologically identified at Chi-Mei Medical Center. Paraffin-embedded oral tissue sections were deparaffinized and rehydrated. For epitope retrieval, slides were boiled in 0.01 M citrate buffer (pH 6.4) in a microwave oven for 20 min and then allowed to cool to RT for 30 min. Subsequent procedures were conducted as previously described [17]. Tissue specimens with more than 10% immunopositive cells were considered as "positive". The intensity of positive staining was classified into three categories: weak, moderate, and strong.

Migration assay. A total of 4×10^4 cells in serum-free culture media were seeded onto the cell migration inserts with 8.0-μm pore size polycarbonate membrane (BD Biosciences, San Diego, CA), and the inserts were then placed into culturing wells that contain media and 10% FBS. Afterward, cells were allowed to migrate for 24 h, and non-migrating cells on the upper side of the membrane were removed with a cotton swab. Migrated cells on the lower side of the membrane were fixed with 100% methanol and stained with Giemsa (Merck, Germany). Migrated cells were counted under bright-field optics at 100× magnification.

RNA interference (RNAi). Custom SMART pool siRNA specifically targeting MAP2A gene (Dharmacon) was used to inhibit endogenous MAP2A expression. Delivery of the siRNA oligos into HaCaT cells was carried out using DharmaFECT 1 transfection reagent according to the user's protocol (Dharmacon). Briefly, HaCaT cells at 70% confluence were transfected with 100 nM MAP2A siRNA or irrelevant dsRNA. After transfection (48 h), the cells were assayed by Western analysis or migration assay.

Statistics. All experimental values were expressed as means ± SD. Statistical analysis was performed using Student's *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison test. For Student's *t*-test, statistical significance was defined as $p < 0.05$. For ANOVA