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Title: Inhibition of Matrix Metalloproteinase-2 modulates malignant behaviour of oral squamous cell carcinoma cells.

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Abstract

Background: Matrix metalloproteinases (MMPs) play a crucial role in the malignant phenotype of cancer cells. In particular, active levels of MMP2 in cancer cells have been associated with invasion and metastasis through the degradation of basement membrane extracellular matrix proteins. However, little is known about the role of this potential biomarker in oral cancer. Our aim was to investigate the effect of MMP2 inhibition on OSCC activity in vitro, as well as to assess MMP2 dysregulation in oral cancer samples.

Methods: Human OSCC cell lines H357 and H400 were tested with: the selective MMP2 inhibitor ARP101 and the MMP2 neutralising monoclonal antibody MA5-13590 to assess cell proliferation in vitro using MTS assay. Cell migration at 12/24 hours was assessed using a Transwell migration assay. Cell invasion was assessed at 24 hours using a Corning Matrigel invasion assay. MMP2 expression was assessed in 208 tissue samples (related to 60 OSCC cases and 9 normal control) using tissue microarray (TMA) and further analysed via TCGA.

Results: Both ARP101 and MA5-13590 monoclonal antibody reduced cell proliferation in both the cell lines tested. Treatment with 4μg/ml of MMP2 monoclonal antibody showed a significant decrease in cell migration at 24 hours. The administration of ARP101 and monoclonal antibody to H357 and H400 cell lines induced a drastic reduction in cell invasion at 24 hours compared to the control In patients, TCGA analysis demonstrated that oral cancer tissues express significantly higher levels of MMP2 mRNA compared to normal oral tissues. Further, IHC analysis on TMA showed significant difference in MMP2 protein expression between low and high histopathological grade OSCC.

Conclusions: We have demonstrated, for the first time, that MMP2 inhibition affects oral cancer cells ability to survive, migrate and invade *in vitro*. Differences between MMP2 expression in normal and malignant tissues varied. Further research on the role of MMP2 in OSCC and novel mechanisms to inhibit MMP2-dependent pathways should be encouraged.

Introduction

Oral cancer is the sixth most common malignancy in the world[1] with oral squamous cell carcinoma (OSCC) encompassing about 90% of oral cancers.[2] Various biomarkers are currently emerging with their importance in treatment options and disease management in patients with oral cancer. One of these biomarkers are MMPs, which are zinc-dependent proteolytic enzymes involved in remodeling the extracellular matrix (ECM). MMPs are also thought to play a major role in other cell behaviours such as cell proliferation, differentiation, angiogenesis, apoptosis and host defense. Whilst all integral to physiological tissue remodeling, among the human MMPs, MMP2 is a type IV collagenase well-known to be involved in cancer pathology,[3] including head and neck cancer.[4]

MMP-2 is secreted as an inactive proprotein and needs to be activated in order to be biologically active and its activation requires proteolytic processing. Interestingly, active levels of MMP2 in cancer cells are highly associated with metastasis through the degradation of basement membrane ECM proteins.[5] Additionally, MMP2 has been shown to proteolytically activate TGF-β, which in turn can promote epithelial mesenchymal transition (EMT), a crucial process involved in cancer metastasis.[6]

So far, clinical trials using MMPs inhibitors as anti-cancer agents have led to contrasting results. This is likely due to the fact that MMPs play complex roles in tissue formation and cancer progression, and it is likely that many MMPs can have both pro and anti-tumorogenic properties.[7] Recently, we have shown that MMP-2 is activated in cancer-associated fibroblasts conditioned medium and caused epithelial dis-cohesion with subsequent increased invasion of keratinocytes in vitro in a TGF-β-dependent manner.[8] Showing a promising significance to the future of cancer therapies, further investigation regarding MMPs role in OSCC and mechanisms to inhibit them is crucial.

The aim of the present study was to investigate the effect of MMP2 inhibition through the use of MMP2 neutralising monoclonal antibody and a selective chemical inhibitor in malignant oral keratinocytes in vitro; as well as to assess the difference between MMP2 expression in normal and malignant tissues through bioinformatic analysis and immunohistochemically through tissue microarray staining. Our results have clearly demonstrated, for the first time, that MMP2 plays a crucial in oral cancer cells ability to proliferate/survive, migrate and invade. Although, differences between MMP2 expression in normal and malignant tissues varied. Future research would benefit from the use of a larger sample, and therefore should be encouraged.

Materials and methods

Cell lines

Two different human malignant oral keratinocyte cell lines H357 and H400 and the normal human oral mucosal epithelial cell line OKF6 derived from different intra-oral sites were selected for the study. The OSCC adherent cell lines were established at Bristol Dental School, University of Bristol, UK by Prime *et al*,[9] from primary explants of tongue and alveolar process squamous cell carcinoma, respectively. All of the OSCCs were HPV negative and were authenticated prior to commencing the experiments.

Culture conditions

The OSCC cell lines were cultured in 100-mm Petri plastic dishes (Corning® 430167, Corning, NY, USA) and grown to 60-80% confluence before being further sub-cultered. Cells were cultured using Dulbecco's Modified Eagle's Medium DMEM (D5796) and nutrient mixture F-12 Ham (N6658) in a 1:1 ratio (Sigma-Aldrich, Castle Hill, NSW, Australia), supplemented with 10% Fetal Bovine Serum (FBS) (SFBS-F, Bovogen, Keilor East, Vic, Australia), 1% penicillin streptomycin mixture (P4333, Sigma-Aldrich, Castle Hill, NSW, Australia), and 0.5 µg/ml hydrocortisone (HC) (H6909, Sigma-Aldrich, Castle Hill, NSW, Australia) in a humidified atmosphere at standard conditions (5% CO_2, 37°C). Epithelial cells grown to 80% confluency were subsequently detached via a pre-treatment of 10 mM EDTA for 10 minutes, followed subsequently with incubation with a 0.25% trypsin in 1 mM EDTA solution (T4049, Sigma-Aldrich, Castle Hill, NSW, Australia) for 5 minutes. OKF6 were cultured as reported in the Supplementary material. The viability of the keratinocytes was confirmed by Trypan Blue exclusion (Trypan Blue Dye, 0.4% solution, 1450021, Biorad, UK).

Gelatin zymography

Activities of MMP2 and MMP9 in H400 cells treated with 10µM and 20µM ARP101, and 2µg/ml neutralising antibody MA5-13590 were determined by gelatin zymography. H400 cells were seeded in 12-well plates with a density of 7.5×104 cells/well and cultured using complete medium (as per above) in a humidified atmosphere at standard conditions (5% CO_2, 37°C). After a period of 24 hours cells were washed twice with PBS and placed in serum-free medium with no antibiotics and incubated for an additional 24 hours. The cells were then treated with 10µM and 20µM ARP101, and 2µg/ml neutralising antibody MA5-13590, and after 24 hours the conditioned media were collected. Total protein content was normalized using Quick Start™ Bradford Protein Assay (Cat# 500020 BioRad). A same amount of protein containing conditioned media was added under non-reducing conditions on an Invitrogen™ Novex™ 10% Zymogram Plus (Gelatin) Protein Gel (Cat. # ZY00102BOX Thermo Fisher Scientific Scoresby, Australia). Gel Electrophoresis was run at a constant voltage of 100V for 90 minutes. Gels were then placed in Novex™ Zymogram Renaturing Buffer (Cat. # LC2670 Thermo Fisher Scientific Scoresby, Australia) and incubated for 30 minutes at room temperature. After incubation the gel was placed in Novex™ Zymogram Developing Buffer (Cat. # LC2671 Thermo Fisher Scientific Scoresby, Australia) and incubated for 30 minutes at room temperature. This was followed by further incubation of Developing buffer at 37 degrees for 36 hrs with gentle agitation.

Areas of gelatin hydrolyzed by MMPs were visualized as clear zones against blue background by SimplyBlue™ Safestain (Cat. No. LC6060 Thermo Fisher Scientific Scoresby, Australia) using an Invitrogen™ iBright™ Imaging Systems (Thermo Fisher Scientific Scoresby, Australia).

Proliferation assays

The cell lines were treated with the selective MMP2 inhibitor ARP101 (A8356 Sigma-Aldrich, Castle Hill, NSW, Australia) and the MMP2 neutralising monoclonal antibody MA5-13590 (ThermoFisher, Scoresby, Australia).

ARP101 ($C_{20}H_{26}N_2O_5S$), is a known MMP-2 inhibitor and functions as a selective MMP-2 inhibitor, with approximately 600-fold more selectivity for MMP-2 versus MMP-1.[10] In addition, ARP101 induces autophagy and autophagy-associated cell death in several different types of cancer cells. Previous studies have effectively used 10μM in the inhibition of MMP2 in cancer cells.[11-13]

Therefore, MMP2 inhibition was performed using 10μM and 20μM ARP101, and 0.02% dimethyl sulfoxide (DMSO) (D4540, Sigma-Aldrich, Castle Hill, NSW, Australia) alone as internal control as it was the manufacturer's recommended solvent for ARP101. In preliminary experiments, two concentrations of the neutralising antibody MA5-13590 were used (2μg/ml and 4μg/ml) to assess the effect of MMP2 inhibition in malignant oral keratinocytes. Cell proliferation was assessed at 0, 24, 48, and 72 hours (TC10™ Automated Cell Counter, Bio-Rad, UK). At each time point the medium was removed, the cells washed twice with 500µl of PBS before being treated with trypsin-EDTA for the splitting and the subsequent cell counting. Only keratinocytes that remained attached to the wells after the double PBS washing were considered alive. Cell proliferation was then validated using MTS cell proliferation assay kit (ab197010, Abcam) as per manufacturer's instructions. The MTS assay was performed using both H357 and H400 cell lines using 10μM and 20μM ARP101, and 2μg/ml neutralising antibody MA5-13590. Absorbance was measured at 490-500nm at timepoints 0, 24, 48 and 72 hours. All the experiments were performed in triplicate.

Migration assay

In vitro cell migration was assessed at 12/24 hours using a Transwell migration assay (CLS3422, Sigma-Aldrich, Castle Hill, NSW, Australia) to investigate the effect of MMP2 neutralising monoclonal antibody MA5-13590 on migration of H357 and H400 cell lines. A concentration of 4μg/ml of the MA5-13590 and serum-free medium (DMEM only) was used to treat the cells in the upper chamber of the assay. A culture medium with 10% FBS without antibiotic was used in the lower chamber. After incubation in standard conditions (37°C, 5% CO_2) the medium was removed at their respective time points. The cells in the upper chamber were formalin fixed and stained using 2% crystal violet solution. After removal of the non-migrated cells, the migrated cells (5 random fields/well) were observed under an inverted light microscope (EVOS™ FLoid™ Cell Imaging Station, Life technologies) and captured images were analysed to count migrated cells using ImageJ Software (ImageJ v. 1.50i, Wayne Rasband, National institute of Health, USA). All the experiments were performed in triplicate.

Invasion assay

In vitro cell invasion of H357 and H400 cells was assessed at 24 hours using a Corning BioCoat Matrigel Invasion Chamber assay (Corning® 354480, Corning, NY, USA). ARP101 10μM and 20μM, the MA5-13590 at 4μg/ml concentration, and serum-free medium (DMEM only) were used to treat the cells in the upper chambers of the assay. A culture medium with 10% FBS without antibiotic was used in the lower chamber as chemoattractant. After incubation in standard conditions for 24 hours the medium was removed. The cells in the upper chamber were formalin fixed for 2 minutes at r/t, permeabilized with 100% ice-cold methanol for 20 min at r/t, and stained using 2% crystal violet solution. After removal of the non-migrated cells, the migrated cells (5 random fields/well) were observed under a BH2 Olympus microscope equipped with a PMW-10MD Sony camera. Captured images were analysed to count migrated cells using ImageJ Software (ImageJ v. 1.50i, Wayne Rasband, National institute of Health, USA). All the experiments were performed in triplicate.

Analysis of MMP2 expression in TCGA database

Clinical and gene expression data pertaining to OSCC patients in the TCGA was downloaded from the GDC data portal on December 2016. All cases with both clinical and RNAseq data available were included.

Tissue microarray (TMA) immunohistochemical (IHC) staining

The TMA slide (OR208 US Biomax, https://www.biomax.us/tissue-arrays/Oral_Cavity/OR208) was baked on a hot plate (~70 celsius) for 10 minutes and submerged into a mixture containing histolene, ethanol and water. Sodium citrate (pH 6.0) was applied to the slide, boiled for 20 minutes, cooled in room temperature for 10 minutes then washed with dH_2O. Tissue samples in the slide were blocked in 3% H_2O_2 methanol for 15 minutes in room temperature, washed with dH_2 O and PBS, and blocked for 2 hours by 10% horse serum in PBS.

The primary MMP2 antibody (MA5-13590 , ThermoFisher) diluted in a blocking buffer (1:100) was applied to the tissues for 30 minutes at room temperature in a humidified chamber. The TMA slide was washed with PBS (3x) afterwards. The tissues were then treated with secondary antibodies for 30 minutes, following the same protocol as the primary antibodies. AB complex was applied to the tissues for 30 minutes, then DAB for 3 minutes and washed with dH_2O (3x). Lastly, a counterstain with hematoxylin was applied and the TMA slide was dehydrated with ethanol and histolene for mounting.

Analysis of stained TMA slide

The stained tissue microarray slide (OR208 US Biomax) was scanned by the Aperio microscope at the Royal Melbourne Hospital. The scanned image was converted into a readable format and was analysed using the Aperio ImageScope program.

The scanned image with the 40x magnification was used, focusing on the sample of interest so that the entire tissue can be viewed on the screen (around 7x magnification). A circular area within the sample was selected using the pen tool, with the precaution that the amount of tissue included for analysis was maximised. The algorithm for a Positive Pixel Count was then applied, and all the values from the annotations window were inputted in a Microsoft Excel spreadsheet. This process was performed for each of the 208 samples (Suppl. Fig. 1). Upon analysis, the Positive Pixel Count designated the cells within each sample with different colours (blue, yellow, orange and red) based on the amount of MMP2 staining. A blue colour indicated the number of negatives, yellow are weak positives, orange are positives and red are the strong positives. Overall, the strong positives (SP) were considered the most coherent staining, thus these data were used for the statistical analysis.

The following formula was used to quantify the amount of strong positives (SP) in a given area for each sample of the microarray: Amount of SP in a given area (SP/micron)= [SP/total area (mm^2)]/1000.

Statistical analysis of the data were conducted through analysis of variance (ANOVA) test with the software program R Commander. The TNM staging provided by the manufacturer was utilised and the samples were divided into two groups: group 1 are samples in stages 1 -2 and the latter comprising of those in stages 3 - 4. Tumour grade was groups as low (Grade 1) or high (Grade 2+3). Tissue samples that were not stained adequately were classified as outliers and excluded from the analysis. Furthermore, inter-rater reliability was calculated through a one sample t-test using the R Commander software program. The mean (SP/micron) value of the triplicate samples was designated as the mean of repeats (MeanOfRep) . The difference between each sample's mean value and its associated mean of repeats was denoted the difference of repeated mean (DifRepMean). To obtain the percentage of variability within the examiners' analysis, the following formula was used: difference in repeated % = [DifRepMean/MeanofRep]x1000

Statistical analysis

Data was analyzed with IBM SPSS statistical software version 21.0 and R 3.3.3. Continuous data was analyzed by variables using *t* test, ANOVA, and Fisher's exact test and linear models with statistical significance defined as $p < 0.05$. Analysis of the TCGA was conducted in the R software environment. Expression of MMP2 (RNAseq data) was normalized, log transformed, and correlated against various clinical variables of interest. Differences were evaluated with Student's t-test and ANOVA. The median value of MMP2 expression in all tumours was used to divide patients into MMP2-high expressing and MMP2-low expressing groups. Kaplan-Meier plots were used to visualise survival outcomes for each of these groups and significance evaluated by the log-rank test.

It is certified that all experiments were performed in accordance with relevant guidelines and regulations as approved by the Ethics Committee of the University of Melbourne. Ethics approval was obtained for this study (University of Melbourne n. 1340716.1). All the cell lines/strains were derived prior to 2001 and therefore, were not subject to Ethical Committee approval in the UK.

Results

Pharmacological inhibition of MMP2 reduces cell proliferation

Firstly, effective gelatinolytic activity of MMP2 secreted from H400 cell line was evaluated with gelatin zymography and was carried out with ARP101 and monoclonal antibody MA5-13590 treated cells. Administration of 10µM and 20µM ARP101 and 2µg/ml monoclonal antibody MA5-13590 reduced the expression of active MMP-2 hence the decreased gelatinolytic activity in gelatin zymography (Suppl. Fig. 2).

A significant reduction of H357 cell growth was observed in the presence of the selective MMP2 inhibitor ARP101 as early as 24 h after treatment and throughout the experimental period (Suppl. Fig. 3a). At a concentration of 10µM, ARP101 was able to inhibit the proliferation of cells after a 24-hour period compared to the control, showing a marked cytotoxic effect at 48 hours. A similar trend was observed with 20μM ARP101. Comparably to H357, treatment with both 10μM and 20 μM ARP101 caused a significant decrease in H400 cell proliferation after 24 hours, and exerted cytotoxic effects at 48 hour timepoint (Suppl. Fig. 3a). We hypothesized that this marked cytotoxic effect is the direct result of the well-known ability of ARP101 to induces autophagy and autophagy-associated cell death in several different types of cancer cells, beside the fact of being an MMP2 inhibitor.

DMSO induced significantly the proliferation at 12 and 24 h for H400 and suddenly inhibited it at 48 hours.

In H357 cells the MTS assay confirmed significant reduction of H357 cell growth in the presence of both concentrations of ARP101 tested, as early as 12 h after treatment and throughout the experimental period (Fig. 1). On a side note, in the H400 cell line a significant increase in cell proliferation was noted at 48 h when cells were treated with 0.02% DMSO only. A significant reduction in cell proliferation was also noted with 10μM ARP101 at 12h and 48 h, while in presence of 20μM ARP101 a significant reduction was detected after 48 hours (Fig. 1).

A monoclonal antibody targeting MMP2 reduces OSCC cell proliferation

The effect of MMP2 monoclonal antibody MA5-13590 on cell proliferation was assessed in H357 and H400 OSCC cell lines. In H357 cell line, treatment with the MMP2 monoclonal antibody at both concentrations of 2μg/ml and 4μg/ml induced a marked decrease in cell proliferation after 24 hours ($p=0.020$ and $p=0.013$ respectively) in comparison to the control (Suppl. Fig. 3b). Incubation of 48 hours resulted in a statistically significant reduction of proliferation in cells treated with 4μg/ml of antibody ($p=0.018$). In our experimental conditions, a similar trend was seen with 2μg/ml of antibody, however the reduction was not statistically significant compared to control. These results were confirmed with the H400 cell line, where a marked reduction in cell proliferation was observed as early as 12 hours in cells treated with 4μg/ml of MA5-13590 antibody ($p=0.034$). Treatment of H400 cells at both concentrations (2μg/ml and 4μg/ml) resulted in a significant decrease in proliferation ($p=0.0008$ and $p=0.026$ respectively) after 24 hours.

The inhibition on cell proliferation peaked at 48 hours for 2μg/ml and 4μg/ml treatment (Suppl. Fig. 3b).

The validating MTS proliferation assay showed similar trend. In fact, in both the cell lines tested, treatment with 2µg/ml of MA5-13590 antibody exerted significant reduction of cell proliferation as early as 12 hours (Fig. 1).

This antibody-mediated reduction of cell proliferation was negligible in the normal oral keratinocyte cell line OKF6 (Suppl. Fig. 4).

Taken together, these results show that targeting of MMP2 with either pharmacological inhibitors or monoclonal antibody induces a reduction of OSCC cell proliferation.

Treatment with anti-MMP2 monoclonal antibody induces a biphasic, time-dependent effect in OSCC cell migration

The highest dose of 4 µg/ml of MA5-13590 monoclonal antibody was selected to assess OSCC cell migration *in vitro*. Serum-free medium in the upper chamber was used to minimize the effect of cell proliferation. H357 cell line treatment showed a significant increase in cell migration after 12 hours when compared to control, while at 24 hours, this trend was inverted with a significant inhibition of cell migration (Fig. 2). In a similar fashion to H357, the administration of 4 µg/ml MA5-13590 to H400 cell line induced an increase in cell migration at 12 hours compared to the control, even though there was no significant difference. Similarly to H357, treatment with the antibody showed a significant decrease in cell migration after 24 hours (Fig. 2).

Inhibition of MMP2 with monoclonal antibody and ARP101 reduces OSCC cell invasion

ARP101 10µM and 20µM and the highest dose of 4 µg/ml of MA5-13590 monoclonal antibody were selected to assess OSCC cell invasion in vitro.

Serum-free medium in the upper chamber was used to minimize the effect of cell proliferation. H357 cell line treatment with both ARP101 (10µM and 20µM) and the monoclonal antibody showed a significant decrease in cell invasion after 24 hours when compared to control ($p=0.03865$, $p=0.02401$, and $p=0.00124$, respectively) (Fig. 3)

Similarly to H357, the administration of the MMP2 inhibitor and of the monoclonal antibody to H400 cell line induced a drastic reduction in cell invasion at 24 hours compared to the control ($p=0.02419$, $p=0.00703$, and $p=0.01193$, respectively) (Fig. 3).

TCGA analysis reveals MMP2 overexpression in OSCC

Given the possible pathophysiological role of MMP2 in OSCC cells *in vitro*, we investigated whether there was a dysregulation of MMP2 expression in OSCC samples.

Data for 472 HNSCC patients (290 oral cavity) were available on the TCGA. 43 cases had associated normal tissue RNAseq data. There was a significant ($p=0.001$) difference in gene expression between normal and cancer tissues (Fig. 3) and this difference persisted when controlling for tumour site. Specifically, OSCC samples exhibited significantly higher levels of MMP2 mRNA compared to controls. Kaplan-Meier curves showed that MMP2 expression (low *vs* high) in oral cancer patients did not associate with difference in survival (Suppl. Fig. 5).

Levels of MMP2 expression did not correlate ($p > 0.05$ by ANOVA) with gender, TNM stage or tobacco and alcohol risk factors. HPV-positive cases however did show a significant decrease MMP2 expression ($p < 0.05$ by ANOVA).

MMP2 protein levels are dysregulated in OSCC samples

Next, we wanted to confirm whether MMP2 was overexpressed at a protein level in OSCC. The tissue microarray used was from 60 OSCC patients (40 male, 20 female) with a mean age of 56.1 years. The control tissue was from the tongue of 9 patients (5 male, 4 male) with a mean age of 25.5. There was a significant difference in MMP2 expression between LOW and HIGH histopathological grade (Tukey's; $p=0.027$) (Fig. 4A), however no overall difference was seen in MMP2 expression (strong positive/micron) between OSCC and Normal (t-test $p=0.937$). MMP2 expression positivity increased with TNM stages, however there was no significant difference among OSCC samples with different TNM stages (I-IV) (ANOVA; $p= 0.723$) or early (Stage I+II) vs advanced (Stage II+IV) disease (ANOVA; $p=0.827$). OSCC from extension subsite of the maxillary sinus or parotid (Other n=4) had a significantly higher mean MMP2 expression than each of other sub sites or normal (ANOVA; $p<0.001$) (Fig. 4B). One outlier > 250 sp/micron was excluded from graphical representation. and this difference was independent of subsite (linear model; $p<0.001$).

Taken together, these data demonstrate that MMP2 is dysregulated in OSCC and could play a role in oral carcinogenesis.

Discussion

In the present study, we show that specific targeting of MMP2 affects OSCC cell proliferation, migration and invasion *in vitro*. In patients, TCGA analysis demonstrated that oral cancer tissues express significantly higher levels of MMP2 mRNA compared to normal oral tissues. It should be considered that such a difference was not found using IHC of tumour cells in a TMA of 60 OSCC patients. However, this IHC TMA analysis demonstrated showed significant difference in MMP2 protein expression between low and high histopathological grade OSCC, this was largely driven by 2 samples of the maxillary sinus and parotid gland. Collectively, our results demonstrated that MMP2 is dysregulated in oral cancer and could be targeted for the treatment of OSCC.

MMPs have long been considered to be attractive cancer targets, and several different types of synthetic inhibitors have been identified as potential anticancer agents. Only a relatively small number of MMP inhibitors (MPPIs) have been examined in clinical trials, and none of these molecules has been established as anticancer drugs mostly due to their adverse effects.[14] For inhibitors designed specifically for MMP-2 such as BAY 12-9566, initial clinical trials were disappointing and thus contributed to a negative view of MMPs as therapeutic targets.[15] Two main reasons can explain the failure of MMPIs in clinical trials. It has now become apparent that some MMPs have antitumor effects; therefore, the broad-spectrum MMPIs used in the initial trials (e.g. marimastat, prinomastat) might block these MMPs and result in tumour progression. In addition, although MMPs are involved in the early stages of tumour progression, MMPIs were tested in patients with advanced disease, beyond the stage when these compounds could be effective.[16] Consistently, our data show that MMP-2 plays a key role in the early events of oral carcinogenesis.

Thus, as a better understanding of MMP pathobiology and inhibitor pharmacokinetic properties emerged, it has become clear that early MMP inhibitor clinical trials were held prematurely. Further complicating matters were problematic conclusions drawn from animal model studies. The most recent generation of MMP inhibitors, including small-molecule inhibitors and monoclonal antibodies that were used in this present study, have desirable selectivities and improved pharmacokinetics, resulting in improved toxicity profiles.[17] However, it will be important to test these drugs in appropriate clinical settings to exploit their full potential as anticancer drugs.

Finally, the importance of MMP-2 beyond its role as therapeutic cancer target should not be underestimated. Recent data and clinical trials clearly point towards a potential value of measuring circulating or tissue MMP-2 levels as diagnostic or prognostic tools, or as a useful secondary outcome for therapies against other primary targets.[18]

In summary, current evidence supports the rationale for investigating the role of MMPs, and particularly MMP-2, in the pathophysiology and treatment of cancer.

We have found that MMP-2 expression correlates with histopathological parameters of OSCC severity, however other studies have shown that high expression and/or activity of MMP-2 were linked with poorer survival in OSCC cases. In one study, both MMP-2 and TIMP-2 expressions showed a statistically significant positive correlation with the grades, stages and metastatic capacities of tumors.[19] Mechanistically, it has been shown that S-Phase Kinase Associated Protein 2 (Skp2), a protooncogene involved in the pathogenesis of several malignancies, regulates the expression of MMP-2 and MMP-9 and enhances the invasive potential of OSCC.[20] This suggests that MMP-2 could contribute to the acquisition of the metastatic phenotype of cancer cells. In our study, analysis of TCGA did not reveal a prognostic significance of MMP-2 expression in oral cancer, however other studies have shown that high expression and/or activity of MMP-2 were linked with poorer survival in Head and Neck and specifically oral SCC.[21-23]

Research shows that MMP-2 is secreted by both cancer cells and cells in the tumour microenvironment. In particular, we have demonstrated that MMP-2 is a major constituent of the secretome of senescent cancer-associated fibroblasts (CAFs) derived from genetically unstable (GU) OSCC.[24] Inhibition of MMP-2 activity was virtually inhibited using an anti-MMP-2 monoclonal antibody, and this resulted in a reduction of migration and invasion of OSCC cells. Here we show that both pharmacological and antibody-mediated blockage of OSCC cell-derived MMP-2 has inhibitory effects similar to those seen after targeting CAF-derived MMP-2. This suggests that targeting MMP-2 can be a valuable anti-neoplastic strategy acting on both the epithelial and mesenchymal components of OSCC.

In addition to synthetic drugs, natural products used in traditional Chinese medicine such as salvianolic acid A (SAA) have demonstrated potential anticancer effects via MMP-2 inhibition. In one study, SAA inhibited the invasion and migration of OSCC by inhibiting the c-Raf/MEK/ERK pathways that control MMP-2 expression.[25] Similarly, extracts of Rubus idaeus, a major constituent of raspberries, have been shown to exert an inhibitory effect of migration and invasion in oral cancer cells and to alter metastasis by suppression of MMP-2 expression through FAK/Scr/ERK signaling pathway.[26]

An interesting finding of our study was the biphasic effect of MMP-2 inhibition on OSCC cell migration. Specifically, a transient increase of migration was observed after 12 hrs of incubation with the monoclonal antibody MA5-13590. This was surprising because inhibition of MMP-mediated shedding of cell-cell and cell-ECM adhesion molecules would be expected to reduce migration and invasion.[6,24,27] One explanation could be that the reduced cleavage of collagen-binding integrins resulting from MMP-2 inactivity is initially balanced by fibronectin-binding integrins, such as those forming focal adhesion complexes. The latter are short-living structures that mediate invasion and therefore could be responsible for the transient increase in cell migration observed upon MMP-2 blockade. Further studies are needed to address this hypothesis.

In conclusion, our results show that MMP2 inhibition reduces OSCC proliferation, migration and invasion. Differences in MMP2 expression in between normal and malignant tissues varied, but were overall suggestive of poorly differentiated tumors associated with MMP2 overexpression. Further research regarding the role of MMP2 in OSCC and mechanisms to inhibit this molecule should be encouraged.

Limitations of this study: There are several limitations to the study. First, this was an in vitro study, carrying all the fundamental problems such as the artificial nonphysiological conditions cells are maintained in (e.g. not reflecting the body temperature of animals, the blood electrolyte concentrations of species, the extracellular matrix or the extent of cell contacts, etc. In addition, within in vitro models cell densities are less than 1% of the tissue situation, and this impairs intracellular signaling. Secondly, we have to acknowledge the limitations of working with TCGA data, such as the inability to map genetic and protein changes to the single cells or distinct cell populations within the tumour.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legend

Figure 1: MTS assays: the effect of 0.02% DMSO, 10 μM and 20 μM ARP101 and 2μg/mL neutralising antibody MA5-13590 on H357 and H400 cells proliferation. Statistical significance is given as follows: $*p < 0.05$; $**p < 0.005$.

Figure 2: Migration assays: migrated cell count of H357 and H400 cells incubated with 4μg/mL neutralising antibody MA5-13590. The results were representative of 3 independent experiments. The representative microscopic fields showed (digital white light microscopy, x460 magnification) are showing H357 and H400 migrated cells at 12 hours and 24 hours timepoints when treated with 4μg/mL (Ab) and with no treatment (Contr). Statistical significance is given as follows: $*p < 0.05$; $**p < 0.005$.

Figure 3: Invasion assays: invaded cell count of H357 and H400 cells incubated with 10 μM and 20 μM ARP101, and 4μg/mL neutralising antibody MA5-13590. Images were taken after 24h at x20 magnification (optical white light microscopy) showing fewer invading cells for treated groups compared to control (representative fields). Graphs show quantification of invasion assays. The results were representative of 3 independent experiments. Data are represented as mean +/-SD. Statistical significance is given as follows: $*p < 0.05$; $**p < 0.005$ as determined by student's t test

Figure 4: Log transformed expression values of MMP2 in the TCGA, comparing normal tissue to tumour. $p=0.001$ by Student's t-test.

Figure 5: Analysis of MMP2 expression using TMA. **a)** MMP2 expression (sp/micron) by tumour grade showed a difference between Low and High

histopathological grade. **b)** MMP2 expression (sp/micron) by site of OSCC sample showed significantly higher scores in tumour samples from maxillary sinus and parotid gland extensions (Other n =4) from all other sites (Tukey $p<0.001$).

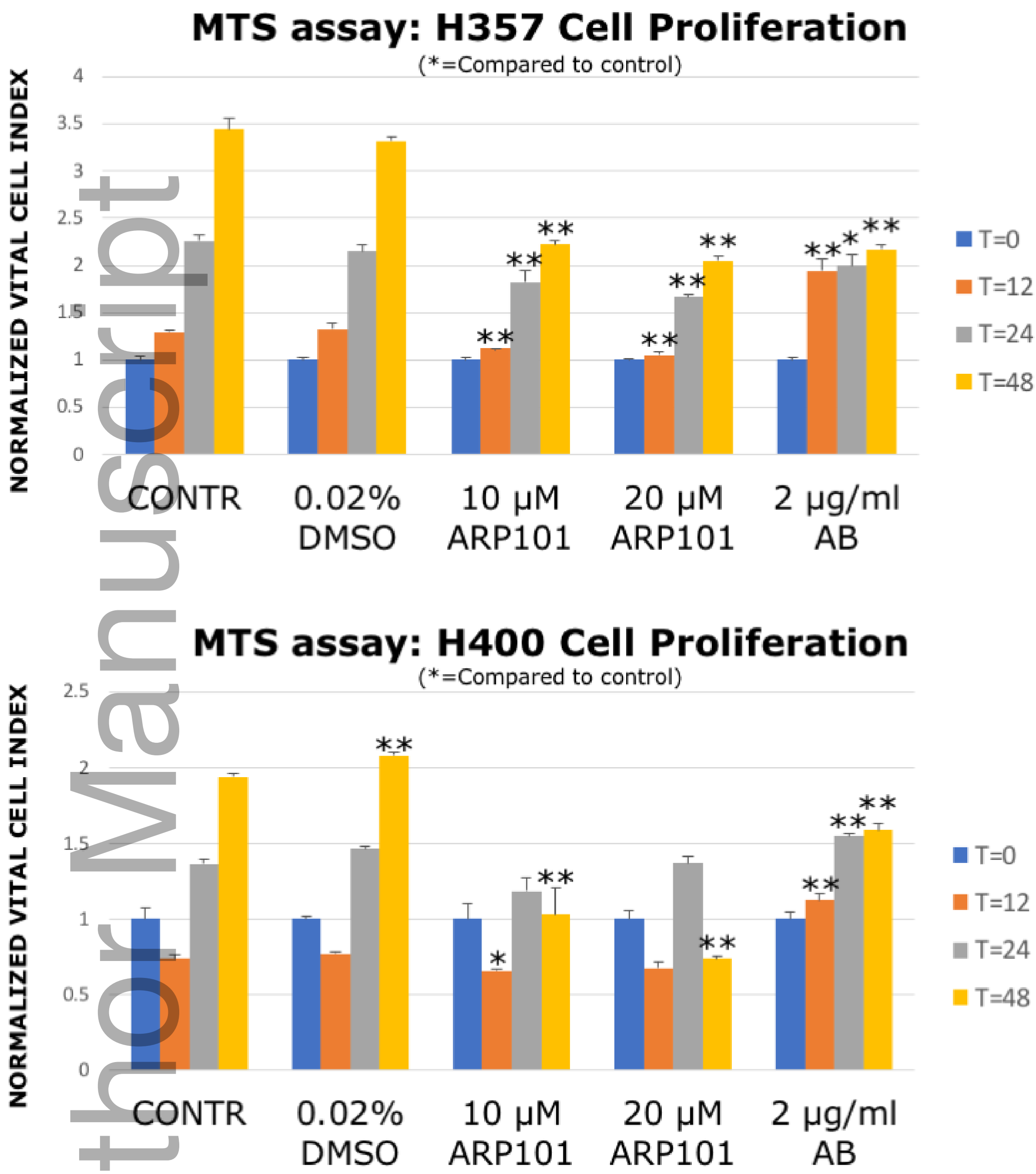
Supplementary Figure 1: a) Random OR208 TMA normal and malignant tissue samples. Positive pixel count was applied (blue = negatives, yellow = weak positives, orange = positives, and red = strong positives). **b)** OR208 TMA slide stained with MMP2 monoclonal antibody (MA5-13590, ThermoFisher)

Supplementary Figure 2: Gelatin zymography of conditioned media: DMEM medium supplemented by 10%FBS was used as positive control (Pos Ct); Conditioned media from H400 cells (lane 2), or supplemented with 0.02% DMSO (lane 1), or 10 μM ARP101 (lane 3), or 20 μM ARP101 (lane 4), or 2μg/mL neutralising antibody MA5-13590 (lane 5).

Supplementary Figure 3: a) Vital cell count of H357 and H400 cells incubated with 10μL DMSO, 10 μM and 20 μM ARP101. Only adherent and vital cells were counted at 0/24/48/72 hour time points. Statistical significance is given as follows: $*p < 0.05$; $**p < 0.005$. b) Vital cell count of H357 and H400 cells incubated with 2μg/mL and 4μg/mL neutralising antibody MA5-13590. Only adherent and vital cells were counted at 0/24/48/72 hour time points. Statistical significance is given as follows: $*p < 0.05$; $**p < 0.005$

Supplementary Figure 4: Vital cell count of OKF6 cells incubated with 10 μM and 20 μM ARP101, and with 2μg/mL and 4μg/mL neutralising antibody MA5-13590.

Supplementary Figure 5: Kaplan-Meier survival plot comparing TCGA patients with high MMP2 expression against those with low MMP2 expression, with mean expression value serving as the threshold. There is no statistically significant difference in survival between the two groups.



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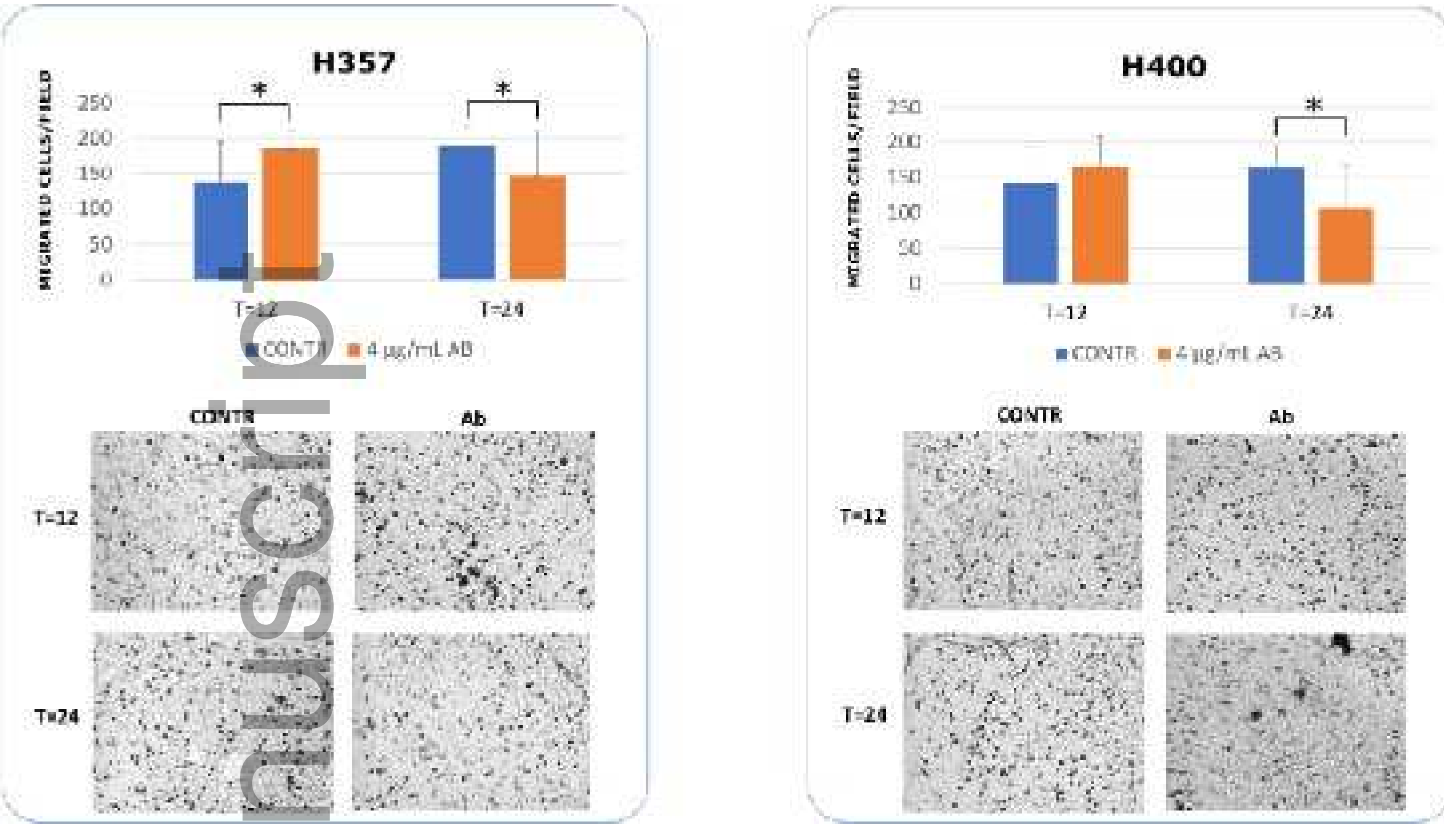

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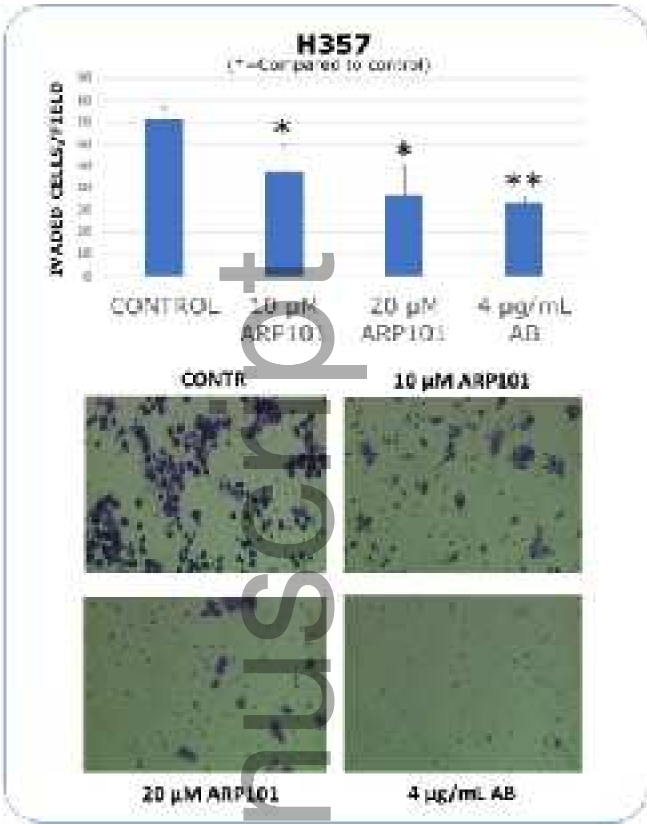

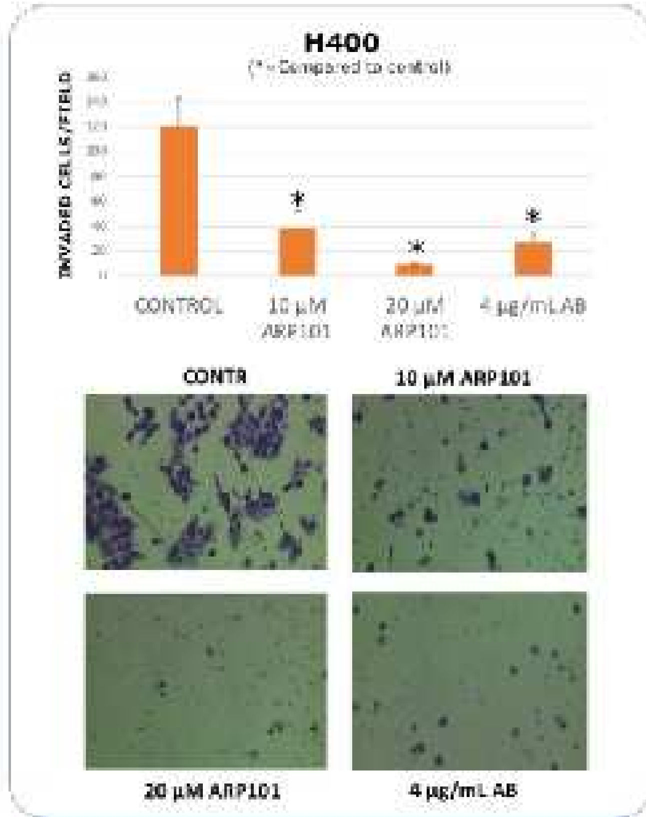

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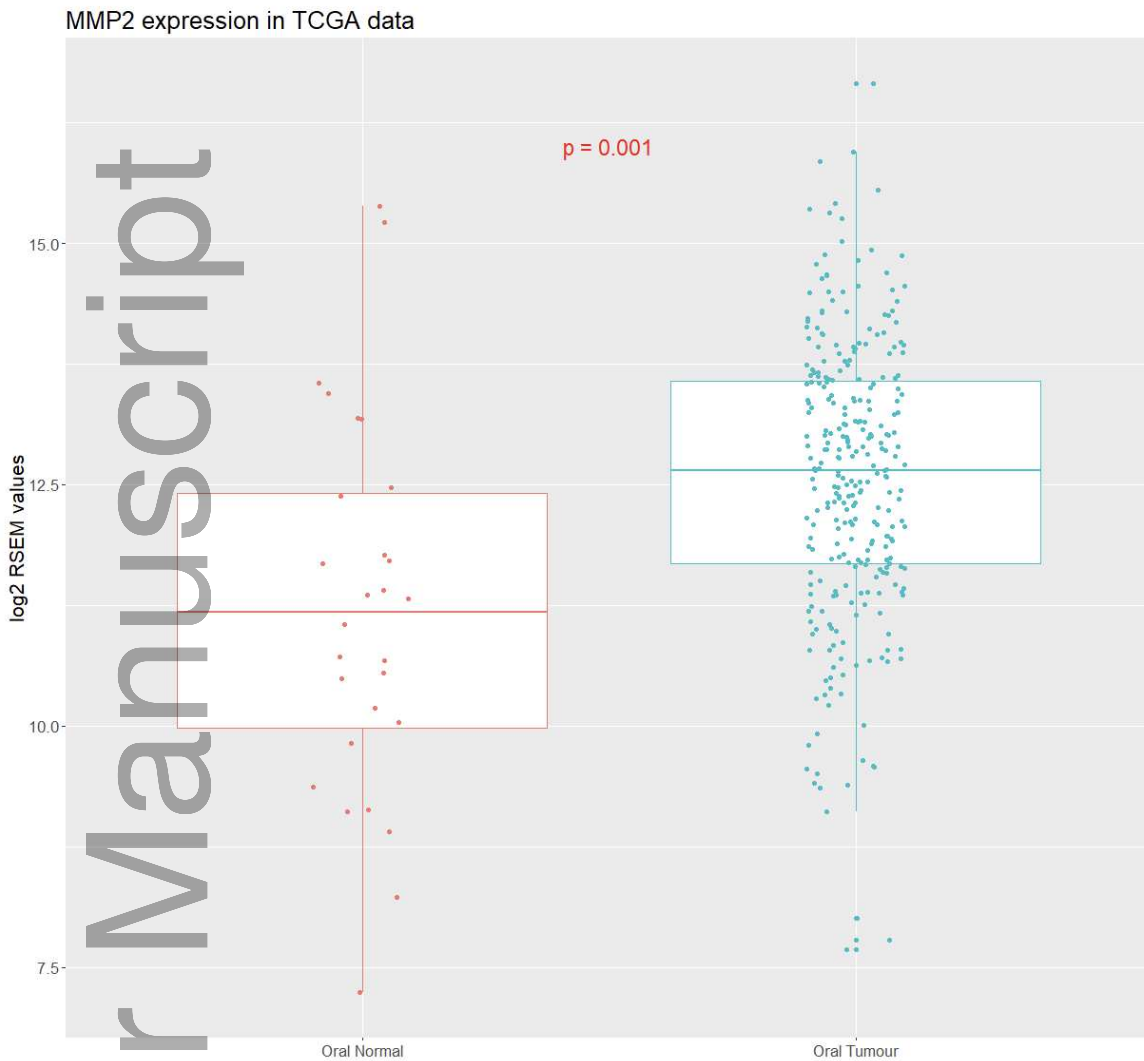

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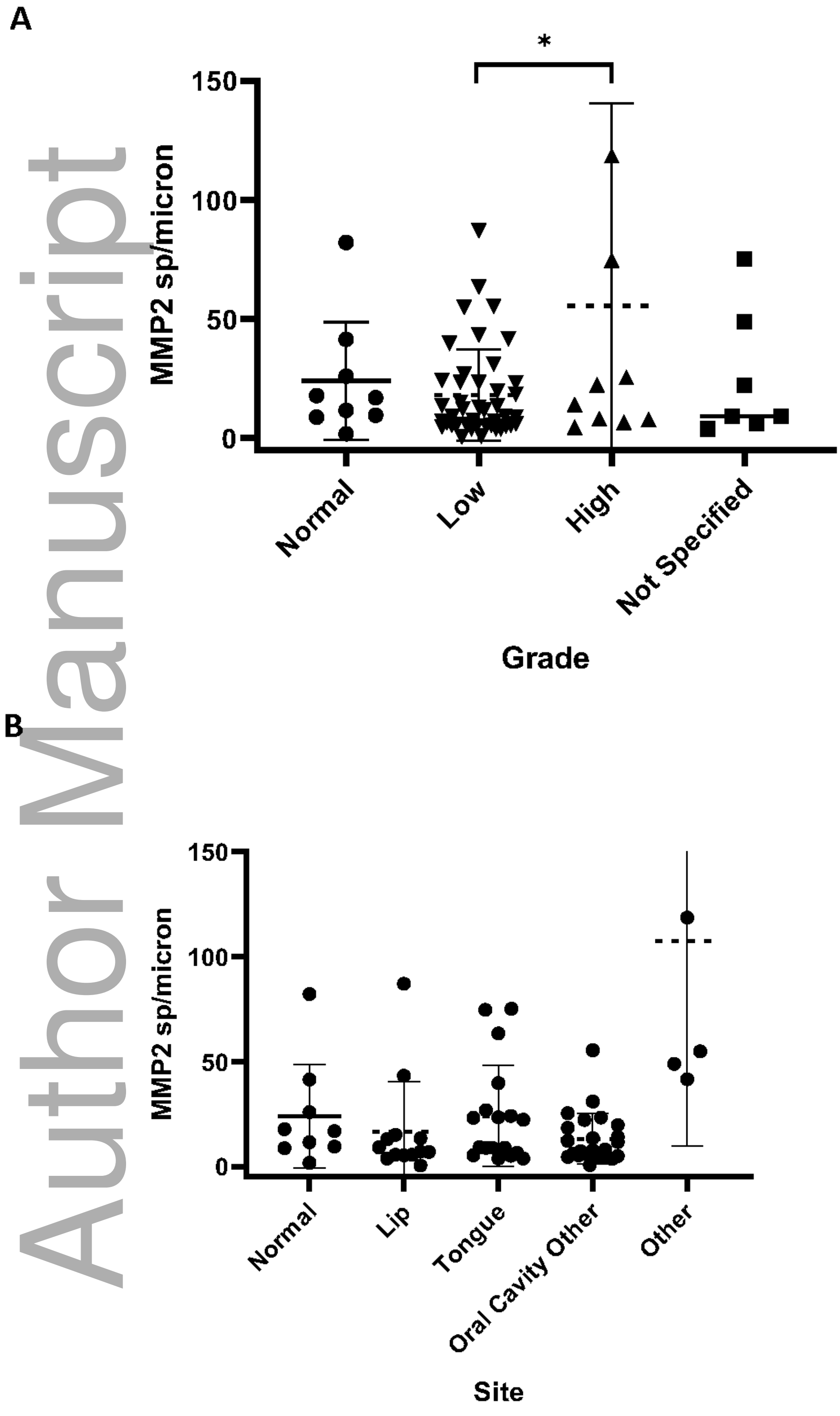

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