Oral cancer is characterized as prolonged exposure to potential risk factors, including tobacco use or cigarette smoking. However, the underlined mechanism of tobacco leads to cancer is still largely unknown. To shed light on the molecular basis of tobacco-induced oral carcinogenesis, we established three sublines of oral cancer cells chronically treated with nicotine (IC50 dose for 3 months). Phenotypic characterization showed that the sublines were tolerant to nicotine, increased cell invasion and migration abilities, along with the altered expressions of epithelial-mesenchymal transition markers. The transcriptomic profiling analysis was performed between parental and the subline cells to global survey and identify nicotine induced genes. Heretical clustering analysis reveals that 1004 genes were differentially expressed, in which 28% of the genes associated with cell mobility. Total of 13 genes were confirmed by RT-PCR assay that over-expressed in the nicotine sublines. ASAP1 was significantly increased in all 3 cell lines, and it was selected for further study. Knockdown ASAP1 expressions by shRNA reduced nicotine induced cell migration (to 60% ~ 80%) and invasion (to 55% ~ 60%) abilities. This ASAP1 silencing further inhibited nicotine- induced MMP2 enzyme activity. Clinical study also supported this finding that ASAP1 was significantly elevated in the oral cancer tissues from patients with smoking habits (P=0.042) but not in the cancer patients without smoking (P>0.714). We therefore concluded that tobacco contributes to oral cancer by promotion of cell invasion and mobility, which was at least in part, through ASAP1 associated signaling pathway.

### Material and method

- **Nicotine treatment**
- **Transcriptome profiling**
- **Verify candidate gene**
- **Knockdown ASAP1 expression**
- **RNA extraction & RT-PCR**
- **shRNA cloning & transfection**
- **Functional assay**
- **RT-PCR & Immunohistochemistry**

### Result

- **Fig. 1**: Dose-dependent effect of cellular viability caused by nicotine in oral cancer cell lines. Cells were exposed to nicotine at the indicated concentrations for 24 and 48 hours. The nicotine dose determined from 1 to 5 mM depended on cell viability.

- **Fig. 2**: Nicotine induced cell migration ability. (A) Nicotine induced spindle-like morphology in SAS and OECM-1 cells. (B) RNA and OECM-1 cells were treated with various dose of nicotine then observed cell migration ability by wound healing assay. Nicotine promoted migration ability in dose dependent manner.

- **Fig. 3**: Nicotine enhanced cell invasion ability. Cells were treated with nicotine then observed cell invasion ability by Matrigel invasion assay. Nicotine induce invasion ability in (A) SAS and (B) OECM-1 cell in a dose dependent manner. Left panel has shown invaded cell stain with crystal violet, right panel has shown quantitative result.

- **Fig. 4**: Functional classification of nicotine related genes. Functional distribution of differentially expressed gene in nicotine treated cells. 28% differential expressed genes participate in cell mobility and angiogenesis.

- **Fig. 5**: RNA expression of ASAP1 gene was verified by RT-qPCR. ASAP1 gene was measured the mRNA expression with nicotine or non-nicotine treatment by RT-qPCR in SAS, OECM3 and C9 cell lines.

- **Fig. 6**: Determination of ASAP1 protein expression in response to nicotine treatment by Western blotting analysis.

- **Fig. 7**: Nicotine enhanced oral cancer cells migration ability through ASAP1. (A) Efficacy of ASAP1 knockdown by shRNA silencing (sh1192 and sh2294) in OECM-1 cells. The cell migration ability were induced after nicotine treatment. When ASAP1 was knockdown, the migration ability were reduced 50-70% than the control vector.

- **Fig. 8**: Nicotine increased oral cancer cells invasion ability through ASAP1. ASAP1 gene knockdown suppressed nicotine induce cell invasion ability both in (A) SAS and (B) OECM-1 cells. Left panel has shown invaded cell stain with crystal violet, right panel has shown quantitative result.

- **Fig. 9**: ASAP1 silencing inhibited migratory phenotype induced by nicotine in dose dependent manner. The cell migration ability induced by Nicotine at 0.35 and 1 mM was in dose dependent manner. knockdown ASAP1 expression will abolish this influence.

- **Fig. 10**: MMP2 regulated the cell invasion phenotype induced by nicotine treatment. (A) Gelatin zymography assay shown MMP2 activity elevated in nicotine treated cells. (B) Silencing ASAP1 expression will reduce MMP2 activity compare to vector cells. Total protein were stained by Ponceau S. shown as loading control.

- **Fig. 11**: ASAP1 up-regulated in oral cancer patient with smoking behavior. Clinical investigation of the ASAP1 gene expressed in health normal (n=5), tumor without smoking (n=9) and tumor with smoking (n=14). ASAP1 expression was significantly increased between tumor and normal group (P=0.0425) or tumor without smoking group (P=0.0356)

- **Fig. 12**: A model for Nicotine-ASAP1-MMP2 pathway. Nicotine induce ASAP1 over-expression and activate MMP2 activity then promote migration and invasion in oral cancer cells.

### Conclusion

1. Nicotine induce cell morphology change and increase cell invasion - migration ability in dose dependent manner.
2. ASAP1 gene has significant increased expression and correlated with our microarray data.
3. Nicotine promote cell migratory and invasion through ASAP1 regulation in dose dependent manner.
4. Nicotine elevate MMP2 activity and promote cell invasion ability. ASAP1 also acts as a upstream regulator to modulate MMP2 activity.
5. ASAP1 was significantly elevated in the oral cancer tissues from patients with smoking behavior.