Effects of *Lactobacillus salivarius* on oral cancer cell proliferation and apoptosis in vitro

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ABSTRACT

Oral cancer is a serious and growing problem in many parts of the world. Probiotic was suggested as a prophylactic measure in many cancers including oral cancer. The aim of this study was to investigate the possible inhibitory mechanism of *Lactobacillus Salivarius Ren* on oral cancer cells (TCA-8113). The effect of *L. Salivarius* Ren on cell proliferation, apoptosis, Expression of COX-2 were also assessed. The results showed that L. Salivarius Ren suppressed cellular proliferation accompanied by enhanced apoptosis, down regulated the COX-2 mRNA levels and protein expression significantly. The results from this study suggest that L. Salivarius Ren exhibits a marked antitumor effect. One of the antitumor mechanisms of *L. Salivarius* Ren may be that its inhibition of COX-2 led to reduced proliferation and induction of apoptosis.

1. Introduction

Oral cancer is a serious and growing problem in many parts of the world. It is estimated that each year there are over 484,000 people diagnosed with oral cancer in the world and approximately 261,000 people die of this disease (Jemal et al., 2011). In China, over 11,900 cases of oral cancers are diagnosed each year and approximately 5,000 patients die of the disease (Han et al., 2010). It is generally believed that oral mucosal carcinomas are predominantly caused by chemical carcinogens such as tobacco, excess consumption of alcohol and betel quid usage (Negri et al., 1993). Despite recent advances in surgery, chemotherapy and radiotherapy, the survival of patients with oral carcinoma remains poor. Furthermore, second primary oral tumors occur rather frequently, which cannot be predicted reliably in the individual patent (Zini et al., 2010). Therefore, the promising approach to reduce the occurrence and development of this malignancy is prevention. Dietary factors play an important role in human health and in the development of certain chronic diseases including cancer.

The suppression of proliferation and inhibition the over-expression of Cyclooxygenase-2 (COX-2) (Harris et al., 2007; Fong et al., 2008) are two most important mechanisms involved in the prevention. In addition, selective COX-2 inhibitors have been reported to suppress COX-2 activity, proliferation activity, and PGE2 production in cancer cell lines (Pandey et al., 2008). However, some of selective COX-2 inhibitors

were reported to be associated with a significant increase in the risk of myocardial infarction and with an increase in the risk of death from cardiovascular causes (Sporn et al., 2005). Therefore, a new strategy of prevention may be essential in the future.

The term probiotic refers to live microorganisms that survive passage through the gastrointestinal tract and have beneficial effects on the host (Parvez et al., 2006). The list of healthful effects attributed to probiotic bacteria is extensive. Epidemiologic and experimental studies suggest that the consumption of the probiotics or fermented milk products could decrease the incidence of certain types of cancer. The vast majority of studies on the anticancer effects deal with colorectal cancer (CRC) (Yamazaki et al., 2000; Caderni et al., 2003), although there are some studies on bladder and oral cancer (Biffi et al., 1997; Zhang et al., 2013). The precise mechanisms by which probiotics exert their anti-CRC are uncertain but might involve preventing the DNA damage induced by carcinogens, modulating the key biomarkers which can inhibit or promote the development of tumor, inhibiting the proliferation or inducing the apootosis of carcinoma cells via its metabolites (Lan et al., 2008; Goldin et al., 1984). However, it has not any reports about inhibition of oral cancer cells of the probiotics.

Lactobacillus Salivarius Ren (*L. Salivarius* Ren) was isolated from fecal samples from healthy centenarians living in villages located in Bama in the Guangxi Zhuang Autonomous Region in China, where has the world's highest longevity ratio. In the previous study, we reported that the *L. Salivarius* Ren could act as potential agents for oral cancer prevention. This is the first report demonstrating the inhibitory effect of the probiotics on oral carcinogenesis (Zhang et al., 2013). In current study, the possible inhibitory mechanism of *L. Salivarius* Ren on oral cancer cell was investigated in TCA-8113 cell line. The effect of *L. Salivarius* Ren on cell proliferation, apoptosis, Expression of COX-2 was also assessed.

2. Materials and methods
2.1. Cell culture

TCA-8113 cell line (derived from human tongue squamous carcinoma) was grown in Dulbecco's Modification of Eagle's Medium (Gibco Co., USA) supplemented with 10% fetal calf serum. The cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2.

Preparation of *L. salivarius* REN cells and its metabolites. After 12h cultivation in Man-Rogosa-Sharpe (MRS) liquid medium (Oxoid), *L. salivarius REN* was harvested by centrifugation (4000g, 10min), washed twice and adjusted to appropriate concentrations in sterile saline (0.9% w/v) for oral administration. The metabolites were collected from the resuspending solution of the strain. This solution was incubated for 1h at room temperature and the water soluble secretion was harvested by centrifugation (4000 g, 10 min) and filtered through a 0.22 μm sterile filter. The concentration of the metabolites was defined as the concentration of the strain in sterile saline.

2.2. TCA-8113 Cell-based assays in general

In cell culture-based assays, TCA-8113 cells were exposed to three different dosages of *L. salivarius* REN metabolites (1, 2, 3×10^9 CFU/ml), *L.casei* metabolites (2×10^9 CFU/ml, negative control) and NS-398 (10mM, Sigma-Aldrich, positive control) for different time, after which COX-2 mRNA expression, protein expression and apoptosis cells were measured.

2.3. Cell Proliferation Assay

Cell proliferation was measured by the (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cells seeded on 24-well microplates at 5×10^4cells/well were treated for 24 h and exposed to 10 μL of MTT solution (5 mg/mL MTT in phosphate-buffered saline (PBS)) for 3 h. Live cells appeared purple in color in response to MTT. After discarding the medium, cells were dissolved in dimethyl sulfoxide. One hundred microliters of cell suspension was transferred to a 96-well plate and cell viability was determined using

VersaMax™ microplate ELISA reader (Molecular Device Co., Sunnyvale, CA, USA).

2.4. Flow cytometric detection of apoptotic cells

TCA-8113 cells were cultured in 6-well cluster plates, and stimulated with different treatment as described above for 24h. Subsequently, the assay was performed with the commercially Annexin V FITC/PI Apoptosis Detection kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Finally, the mixtures were analyzed with a FACSCalibur flow cytometer (BecktonDickson).

2.5. Quantitative real-time PCR

TCA-8113 cells were grown in monolayer and stimulated for 12h with different treatments as described above. After harvest, total RNA was extracted from cells with NucleoSpin RNA II kit (QIAGEN). RT-PCR was done as described24 using COX-2 and β-actin primers obtained from Invitrogen (Beijing). The sequences of the β-actin and COX-2 primers were as follows: β-actin, forward 5'-GGATCCGACTT CGAGCAAGAGATGGCCAC -3' and reverse 5'- CAATGCCAGGGTACATGGTGGTG -3'; COX-2, forward 5'-GCGAGGGCCAGCTTTCACCA -3' and reverse 5'- TTCCCTCAGCCAGATTGTGG CA -3'. Up regulation and down regulation of β-actin and COX-2 were determined by the $2^{-\triangle\triangle Ct}$ method (Pfaffl et al., 2002).

2.6. Immunohistochemistry

TCA-8113 cells were grown in coverslips and stimulated for 12h with different treatments as described above. The coverslips were rehydrated and subsequently incubated with a polyclonal primary antibody (COX-2, 1:50 solution, Cell Signaling Technology, Inc) at room temperature for 1h. The secondary antibody (anti-rabbit-mouse-goat-antibody) was incubated for 15 min at room temperature, followed by incubation with strepavidin-POD (Dako) for 15 min. Antibody binding was

visualized using AEC-solution (Dako). The samples were then counterstained by haemalaun solution (Dako). Slides were subsequently reviewed in a blinded fashion. The immunoreactivity of the samples was graded on the basis of the number of positively stained cells. COX-2 positive cells were counted in eight randomly selected fields at a magnification of 400. The results of the cell counts were given as means of percentages of positive cells of all the cells counted in a defined field.

2.7. Western Blotting

TCA-8113 cells were grown in monolayer, and stimulate for 18h with different treatment as described above. The cells were harvested, washed with cold PBS, and lysed with ice-cold lysis buffer supplemented with protease inhibitors. Cell lysates were analyzed for Western blot analysis using COX-2 antibody (Cell Signaling). Blots were reprobed with actin to compare protein load in each lane.

2.8. Statistical analysis.
Statistical analysis on the incidence of lesions and immunoreactivity COX-2 were performed using Fisher's exact probability test. The data of positive cells ratio in immunohistochemistry was analyzed using the Student's t test. The data of COX-2 mRNA levels and flow cytometry analysis were analyzed using the Duncan test. The results were considered statistically significant if the P value was less than 0.05.

3. Results and discussions

3.1. Anti-proliferative effect of *L. Salivarius* Ren on TCA-8113 cells

Cell proliferation is suggested to play an important role in multistage carcinogenesis, including oral tumorigenesis. Many of possible cancer preventive agents could suppress cell proliferation activity (Versalovic et al., 2008). In the present study, TCA-8113 cell line was treated with three doses of metabolites for 24 hours (Figure 1). After treatment, the proliferation of the cell lines was significantly

inhibited, especially in high dose (3×10^9 cfu/ml). Furthermore, the growth rate of cell lines was greatly decreased by incubation with NS-398 ($P < 0.05$), and the viability of TCA-8113 cell lines treated with artesunate decreased in a dose-dependent manner, whereas the metabolites of *L. casei* had no significant effect on cell proliferation activity ($P > 0.05$).
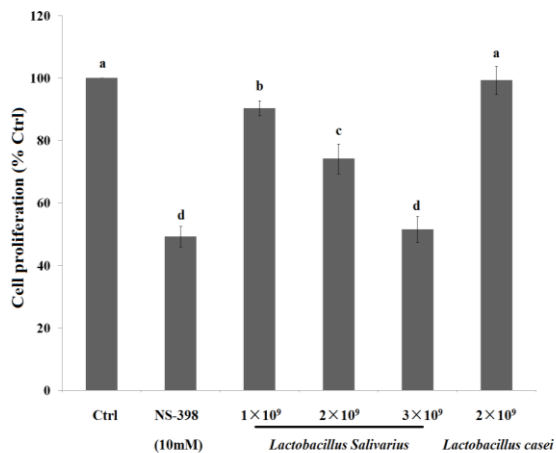

Figure 1. Anti-proliferative effect of *L. Salivarius* Ren on TCA-8113 cells

3.2. *L. Salivarius* Ren induced apoptosis in TCA-8113 cells

The neoplasia inhibition effect of anti-cancer agents is always associated with their ability in inducing apoptosis (Granado-Serrano et al., 2006). To determine whether the inhibitory effects of *L. Salivarius* Ren on TCA-8113 cellular proliferation are accompanied by enhanced apoptosis, we utilized Annexin V FITC/PI Apoptosis Detection kit to detect the different stage apoptosis. The results of flow cytometry analysis of apoptosis were shown in Fig 2. After 24h exposure, the metabolites of *L. Salivarius* Ren could dose-dependently increase the early and late apoptosis cells ratio. Under the treatment of high dose metabolites (3×10^9 cfu/ml), 10.32% of treated cells entered the early apoptosis, and 28.15% entered the late apoptosis. In addition, under the treatment of NS-398, 7.44% of treated cells entered the early apoptosis, and 32.36% entered the late

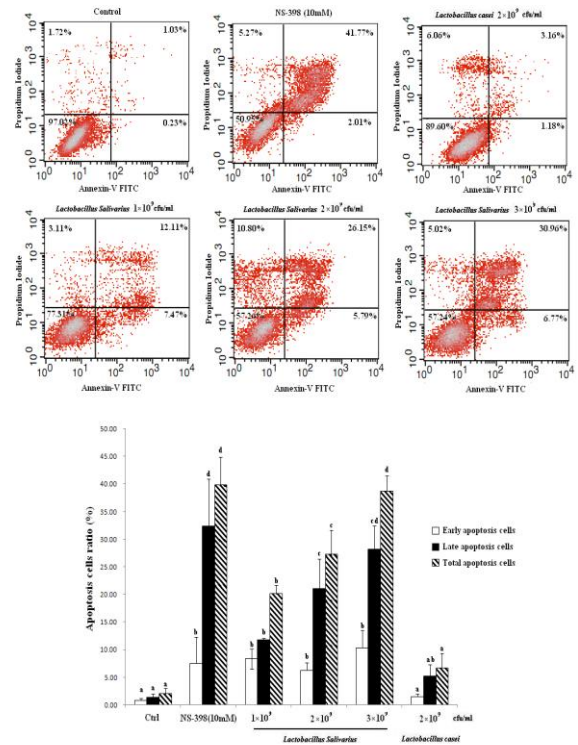apoptosis, whereas the metabolites of *L. casei* had no significant effect on apoptosis cells ratio ($P > 0.05$).

Figure 2. Flow cytometry analysis of apoptosis induced by the metabolites of *L. Salivarius* Ren

3.3 Effect of the metabolites of *L. Salivarius* Ren on COX-2 mRNA levels and protein expression

COX-2 is constitutively overexpressed in a variety of malignancies, including gastric cancer, breast cancer, bladder cancer, non-small-cell lung cancer, and colorectal cancer (Harris et al., 2007; Fong et al., 2008), and COX-2 over-expression is associated with carcinogenesis, progression, invasion, metastasis, and a poor prognosis (Hanahan & Weinberg, 2000; Greenhough, 2000). Therefore, inhibition of COX-2 expression may prevent or reverse gastric carcinogenesis. There is increasing evidence demonstrating that inhibition of expression of COX-2 has antitumor activity against gastrointestinal carcinoma. Given the apoptotic response displayed following *L. Salivarius* Ren treatment, we investigated whether L.

Salivarius Ren treatment reduces the overexpression of COX-2 of TCA-8113 cells in mRNA and protein levels.

The results of RT-PCR (Figure 3) revealed that the metabolites of *L. Salivarius* Ren could down regulate the COX-2 mRNA levels of TCA-8113 cell line significantly ($P<0.05$). After a 12h exposure, an approximately 9-fold decrease in COX-2 transcription was recorded under the treatment of high dose metabolites (3×10^9cfu/ml). NS-398, a selective COX-2 inhibitor, caused an approximately 10-fold decrease, whereas the metabolites of *L. casei* had no significant effect on COX-2 transcription ($P > 0.05$). These results suggested that the modulation of COX-2 by *L. Salivarius* Ren is positively correlated with its mRNA expression, which indicated the regulation of COX-2 by *L. Salivarius* Ren could be at the transcription level.
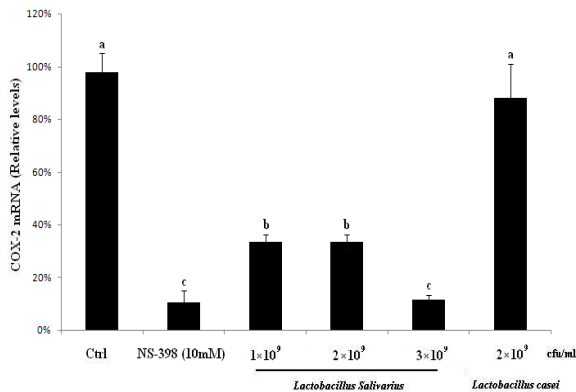

Figure 3. Effect of the metabolites of *L. Salivarius* Ren on COX-2 mRNA levels

The result of immunocytochemistry (Figure 4A, Table 1) revealed that metabolites of L. Salivarius Ren could downregulate the COX-2 protein expression significantly ($P<0.05$).The TCA-8113 cell lines were strong positive immunoreactivity of COX-2. In addition, NS-398 could decrease the immunoreactivity of COX-2, whereas the metabolites of *L. casei* had no significant effect on COX-2 protein expression ($P > 0.05$). We also used the Western Blotting to reconfirm the observation in immunocytochemistry assays. As shown in

Figure 4B, the secretion of *L. Salivarius* Ren down regulated the COX-2 protein expression significantly in a dose dependant manner, whereas those of *L. casei* (positive control) had no significant effect.
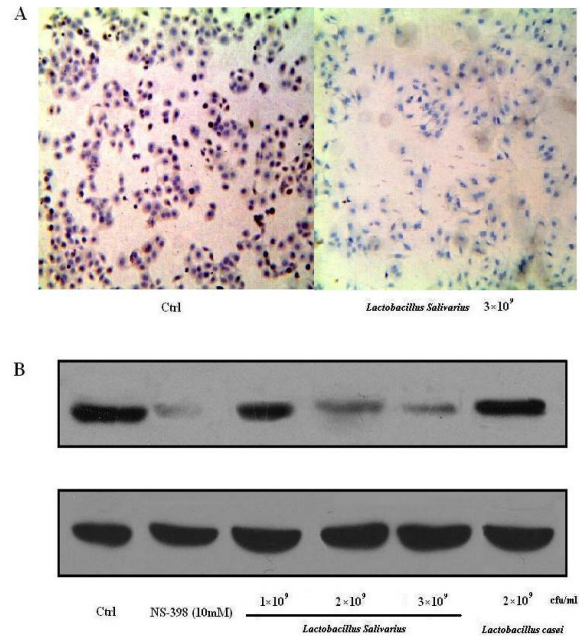

Figure 4. Effect of the metabolites of *L. Salivarius* Ren on COX-2 protein expression

Table 1. COX-2 immunocytochemistry staining

Treatment	COX-2 stainning		
	-	+	+ +
No treatment	0(0%)	1(11%)	8(89%)
NS-398(10mM)	7(78%)[a]	2(22%)	0(0%)[a]
1×10^9cfu/ml *L. Salivarius* Ren	1(11%)	4(44%)	4(44%)
2×10^9cfu/ml *L. Salivarius* Ren	3(33%)	3(33%)	3(33%) [b]
3×10^9cfu/ml *L. Salivarius* Ren	5(56%)[b]	3(33%)	1(11%) [a]
2×10^9cfu/ml *L. casei*	0(0%)	3(33%)	6(67)

4. Conclusions

In summary, the results from this study suggest that *L. Salivarius* Ren exhibits a marked antitumor effect. One of the antitumor mechanisms of *L. Salivarius* Ren may be that its inhibition of COX-2 led to reduced proliferation and induction of apoptosis. This study suggests the possible effectiveness of a

novel preventive approach for oral malignancy by using the probiotics, although further studies will be necessary.

5. References

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