ORAL DISEASES

Oral Diseases (2009) 15, 466–471. doi:10.1111/j.1601-0825.2009.01572.x

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ORIGINAL ARTICLE

The effects of cigarette exposure on rat salivary proteins and salivary glands

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OBJECTIVE: Passive smoking is the involuntary inhalation of cigarette smoke (CS) and has an adverse impact on oral health. We examined the effect of CS exposure on saliva and salivary glands (SGs).

METHODS: Cigarette smoke-exposed rats were intermittently housed in an animal chamber with whole-body exposure to CS until killed. Whole saliva was collected before CS exposure (0 day), and 15 and 30 days after the start of CS exposure. Saliva secretion was stimulated by administration of isoproterenol and pilocarpine after anesthesia. SGs were collected on 31 days.

RESULTS: The increase in body weight of the CS-exposed rats was less than that of the control rats. Salivary flow rates did not differ at 0, 15 or 30 days after the start of CS exposure. However, the amylase and peroxidase activities and total protein content in the saliva were significantly lower in 15-day CS-exposed rats than in 15-day control rats. Histological examination of the SGs of CS-exposed rats showed vacuolar degeneration, vasodilation and hyperemia.

CONCLUSION: These results suggest that CS exposure has adverse impacts on salivary composition and SGs, which could aggravate the oral environment.

Oral Diseases (2009) **15**, 466–471

Keywords: passive smoking; cigarette smoke; saliva; amylase; peroxidase

Introduction

Many studies have reported the adverse impacts that tobacco smoking has on oral health. It was demonstrated that there was a strong positive correlation between cigarette smoking and the increased incidence and severity of periodontal disease (Bergstrom and Eliasson, 1987; Bergstrom, 1989; Gonzalez *et al*, 1996; Tomar and Asma, 2000) and caries (Axelsson *et al*, 1998). Cigarette smoking is a known risk factor for these diseases.

Tobacco smoke includes mainstream and sidestream smoke. Passive smoking is the involuntary inhalation of cigarette smoke (CS). CS includes sidestream smoke and exhaled mainstream smoke. Both mainstream tobacco smoke and CS contain numerous pharmacologically active, toxic and mutagenic chemicals (e.g. nicotine, carbon monoxide, tar, ammonia and particulate) produced by burning tobacco, and it is reasonable to assume that CS exposure might also affect oral health. The relationship between smoking in the household and pediatric dental caries has already been reported by cross-sectional studies (Williams *et al*, 2000; Aligne *et al*, 2003; Shenkin *et al*, 2004).

Saliva plays a significant role in maintaining oral health. It is a key defense against bacteria and viruses. We postulated that CS exposure might alter salivary components and salivary glands, which could contribute to oral health changes caused by CS. In this study, we examined the relationships between CS and salivary function, and salivary gland histology.

Materials and methods

Animals

Male Wistar rats (7 weeks old) were used in this study. The animals were kept in plastic cages with access to food and water *ad libitum*. All 6-week-old animals were allowed to acclimatize to the laboratory environment for 1 week prior to the CS exposure. All animals were weighed daily to monitor growth rates. Rat chow consumption was measured in the CS-exposed group and control group over the 31-day experimental period. Four rats were housed per cage. The daily total amounts of food and water consumption were determined during

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Received 18 October 2008; revised 8 April 2009; accepted 8 April 2009

the experimental period. The total amount of chow eaten over the 31-day experimental period by one rat was calculated as the dairy food consumption per rat. To account for the difference in body weights of the CS-exposed *vs* control animals, relative food consumption was also calculated by dividing the weight of food eaten by the weight of the animal and expressed as gram of food consumption per 100 g of the mean of daily rat body weight during the experimental period (Hoffman *et al*, 2002). The amount of drinking was also measured and calculated.

All procedures for animal care were approved by the Animal Management Committee of Matsumoto Dental University. All animal experiments were performed in compliance with the Guidelines for Proper Conduct of Animal Experiments, established by Science Council of Japan.

Cigarette smoke exposure protocol

The animals were assigned to the intermittent CS exposure ($n = 8$) or control group ($n = 8$). CS exposure was performed according to the method of Nogueira-Filho Gda *et al* (2007). Briefly, CS animals were intermittently housed in an animal CS exposure chamber for 8 min, three times daily, until killing on day 31. The animal tobacco device was composed of a $45 \times 25 \times 20$ cm^3 clear acrylic chamber (CS exposure chamber), two air-pumps and four inflow tubes (Figure 1). Four animals of the CS group were housed in the chamber at the same time, and the smoke of 10 pieces of tobacco, containing 1.4 mg of nicotine and 17 mg tar in each piece, was puffed into the chamber. The CS animals were forced to breathe air containing tobacco smoke for 8 min. The top was covered except for a small opening with a length of 45 mm and width of 6 mm. The control animals were not exposed to the tobacco smoke at any time. The control rats were moved to another cage for 8 min, three times daily, until killing on day 31.

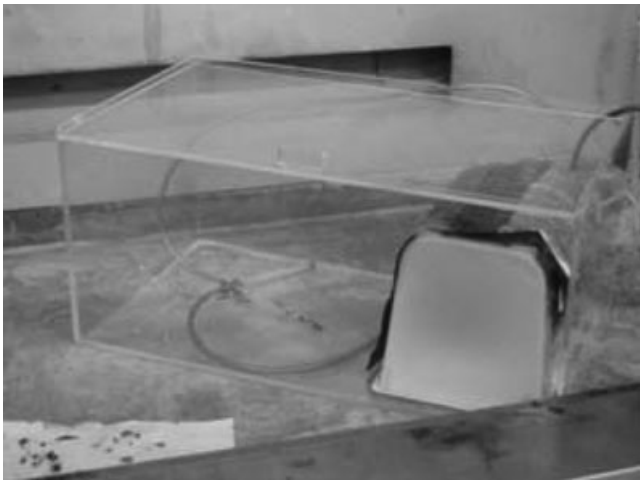

Figure 1 CS exposure device. The acrylic chamber was composed of two subchambers: the cigarette compartment and the animal compartment. Four rats were housed in the chamber at the same time. The rats were exposed to cigarette smoke generated from 10 pieces of tobacco/time, 8 min/time and three times a day in the acrylic chamber

Collection of saliva

Whole saliva was collected before CS exposure (0 day), and 15 and 30 days after the initiation of CS exposure. Saliva was collected at least 8 h after the last CS exposure. Whole saliva was collected from the oral cavity by a micropipette. Before saliva collection, the rats were anesthetized with pentobarbital (40–50 mg kg^{-1}, i.p.). Saliva secretion was stimulated by administration of isoproterenol (2.0 mg kg^{-1} i.p.) and pilocarpine (0.5 mg kg^{-1} i.p.) into CS and control rats (Wang *et al*, 1998). The salivary flow rate was measured over a 15-min period after administration of isoproterenol and pilocarpine.

Assessment of salivary cotinine levels

Saliva cotinine concentrations were measured by a high-sensitivity salivary cotinine quantitative enzyme immunoassay kit (Salimetrics, State College, PA, USA) according to the manufacturer's protocol. Each saliva sample was diluted at 1:10 in saline.

Biochemical assays

Amylase activity was determined using the alpha-Amylase Assay Kit (Kikkoman, Chiba, Japan) according to the manufacturer's protocol. Briefly, in this assay 0.5 ml of matrix reagent and 0.5 ml of enzyme reagent were mixed in a tube and incubated at 37°C for 5 min. Saliva (1:50) measuring 0.1 ml was added into the tube and gently mixed. The tube was incubated at 37°C for 10 min. The reaction was stopped by adding 2.0 ml of stop solution. The optical density was determined at 400 nm. Amylase activity in saliva was expressed as units per milliliter (U ml^{-1}). Amylase output (U per 15 min) was calculated by multiplying the salivary flow rate (ml per 15 min) and the amylase activity (U per ml). All samples from the same rat were assayed (in duplicate) in the same assay to eliminate between-assay error of measurement.

Salivary peroxidase activity was measured using tetramethylbenzidine (TMB). In this assay, saliva was diluted (1:10) with saline and 20 μl was placed into a microplate. Eighty microliters of substrate solution containing TMB was added and the plate was incubated for 30 min at room temperature in the dark. Color development was terminated upon the introduction of 100 μl of 0.6 N sulfuric acid. The optical density of each well was determined with a microplate reader at 450 nm. A standard inhibition curve was generated by serial dilution (1:2) of a solution consisting of 400 mU ml^{-1} human myeloperoxidase (Calbiochem, Darmstadt, Germany) in saline to obtain five dilutions of known concentration.

Histology

Salivary glands were collected 31 days after the initiation of CS exposure. Rats were deeply anesthetized with pentobarbital (50 mg kg^{-1}, i.p.). After decapitation, salivary glands were removed quickly, placed in a

fixative [4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4)] and immersed in the fixative for 1 day. Paraffin sections of the salivary glands were stained by hematoxylin and eosin.

Statistics

Results are expressed as the mean ± standard error. The statistical significance of the difference in body weight gain was assessed by Student's *t*-tests. To determine the significance of differences in salivary cotinine concentration, flow rate, total protein amount, amylase activity and peroxidase activity, two-factor repeated-measure analysis of variance (ANOVA) was performed and Holm corrected *post hoc* paired *t*-tests (0 day *vs* 15 or 30 days) or Welch test (CS *vs* control) were applied.

Results

Body weight gain

Figure 2a shows the body weight of the CS-exposed rats ($n = 8$) and control rats ($n = 8$), which was measured daily during the 31-day experimental period. The body weight gain over the 31-day period was 49.3% in the control rats and 30.1% in the CS-exposed rats. The body weight of the CS-exposed rats was significantly lower than that of the control rats from 1 day to 31 days after the initiation of CS exposure.

Food and water consumption

Cigarette smoke-exposed rats ate less per day during the 31-day experimental period than the control rats (Figure 2c). However, chow consumption relative to body weight was almost the same during the 31-day period in the two groups. The CS rats drank more per day than the control rats (Figure 2b). The amount of drinking water relative to body weight was also higher in the CS rats.

Salivary cotinine level

Cigarette smoke-exposed rats had increased salivary levels of cotinine (Figure 3), the major metabolite of nicotine present in tar. Salivary cotinine levels at 15 and 30 days in the CS-exposed rats were 64.6 ± 8.3 (38.6–100.9 ng ml^{-1}) and 68.5 ± 8.0 (45.4–113.1 ng ml^{-1}), respectively. However, the salivary cotinine levels at 0, 15 and 30 days in the control rats and at 0 d in the CS-exposed rats were less than 1 ng ml^{-1}. Two-factor repeated measures ANOVA showed significant differences in salivary cotinine levels between the CS and the control groups ($F = 112.05$, $P < 0.001$) over time ($F = 36.70$, $P < 0.001$), and the interaction of both factors ($F = 36.84$, $P < 0.001$). These results are consistent with salivary cotinine levels recorded in CS exposure.

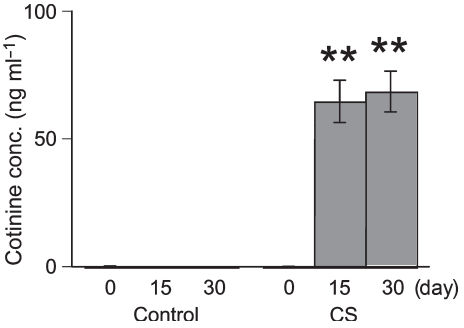

Figure 3 Salivary cotinine levels. The salivary cotinine level of 15-day CS group was significantly higher than those of 15-day control and 0-day CS groups. Similarly, the salivary cotinine level of 30-day CS group was increased compared with those of 30-day control and 0-day CS groups. **Significant difference *vs* 0 day and other group ($P < 0.01$)

Effect of CS on salivary function

The salivary flow rates of the CS and control groups are shown in Figure 4a. Two-factor repeated measures ANOVA showed a significant difference in salivary flow rate over time ($F = 3.52$, $P < 0.05$) but not between the CS and control group ($F = 2.46$, $P = 0.14$), nor their interaction ($F = 1.09$, $P = 0.35$). However, the salivary flow rates of within each group did not differ at each time point (0 day *vs* 15 days and 0 day *vs* 30 days).

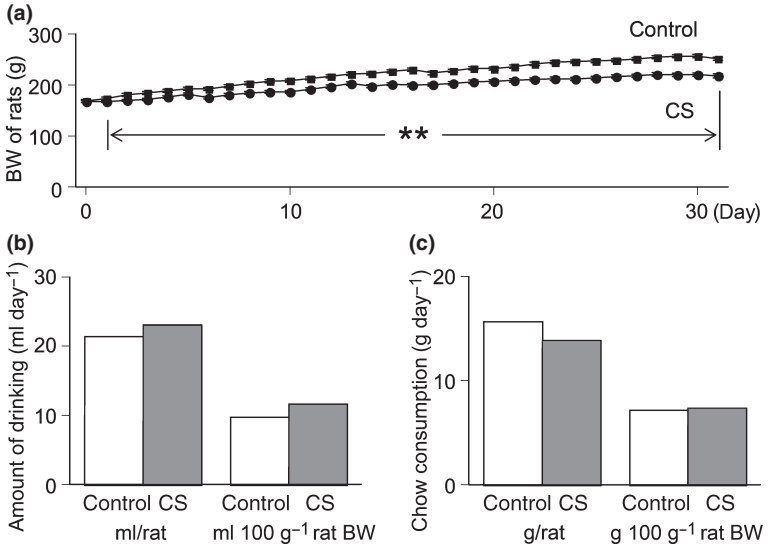

Figure 2 Growth curves of rats exposed to CS. (**a**) The body weights (BW) of rats during the 31 days of CS exposure ($n = 8$) and control rats ($n = 8$) are shown. The amount of increase in body weight of the CS rats was less than that of the controls. The maximum difference in body weight between the CS and control groups was 36.4 g. The body weight of the CS-exposed rats was significantly lower than that of the control rats between 1 and 31 days (**CS *vs* control $P < 0.01$). The mean of water (**b**) and food (**c**) consumption (ml drank or gram eaten per day) per rat and per 100 g of the mean of daily rat body weight during the 31-day experimental period of the CS and control groups is shown

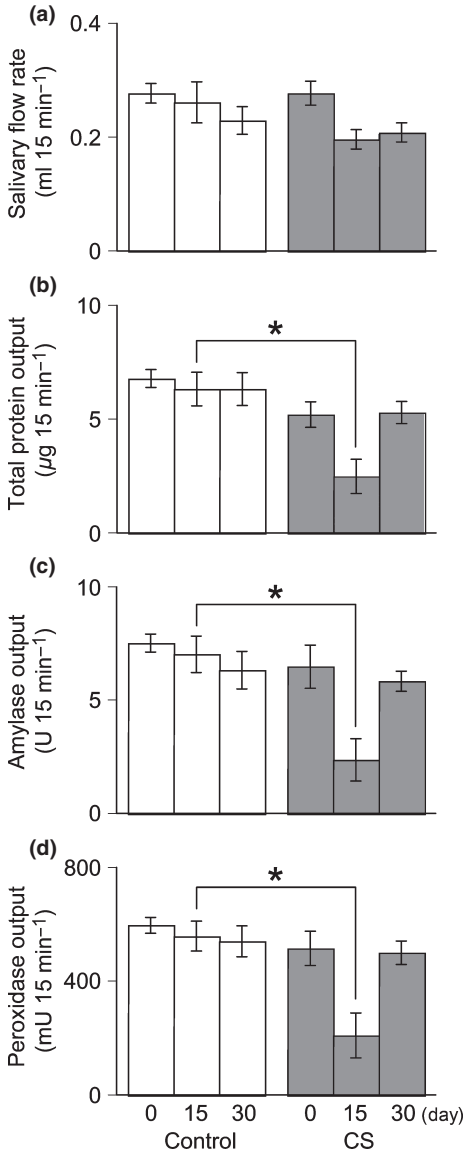

Figure 4 Amount of salivary protein and activity of amylase and peroxidase. (**a**) The salivary flow rate over 15 min did not show differences at 0, 15 and 30 days in either the CS rats or control rats. (**b–d**) The levels of salivary total protein (**b**) and amylase (**c**) and peroxidase (**d**) activities of 15-day CS group were significantly lower than those of 15-day control group. *Significant difference *vs* 0 day and other group ($P < 0.05$)

Two-factor repeated measures ANOVA revealed significant differences between the CS and control group over time and their interaction in total salivary protein (group: $F = 14.73$, $P < 0.01$, time: $F = 4.36$, $P < 0.05$, interaction: $F = 3.18$, $P = 0.06$; Figure 4b), salivary amylase activity (group: $F = 12.03$, $P < 0.01$, time: $F = 4.54$, $P < 0.05$, interaction: $F = 4.34$, $P < 0.05$; Figure 4c) and salivary peroxidase activity (group: $F = 13.71$, $P < 0.01$, time: $F = 5.16$, $P < 0.05$, interaction: $F = 4.40$, $P < 0.05$; Figure 4d). Significant differences in total salivary protein level, salivary amylase activity and salivary peroxidase activity were seen only between 0 day *vs* 15 days in the CS-exposed rats. However, amounts of total salivary protein, and salivary amylase and peroxidase activities were significantly lower in the CS group. These three parameters in the CS 30 days rat saliva recovered to the same levels as those in the control 30 days rat saliva.

Histological changes

As shown in Figure 5b,d, the salivary glands of the CS-exposed rats showed vasodilation and hyperemia. In the parotid grand, the area of the intercalated duct portion (IP) and area of the striated duct portion (SP) were larger in the CS group than in the control group. Vacuolar degeneration (VD) was observed sparsely throughout the entire CS-exposed parotid grand. On the other hand, many VD existed in the central part of the CS-exposed submandibular gland. Inflammatory cells were not infiltrated in the CS-exposed parotid grand and submandibular gland.

Discussion

This study provides *in vivo* evidence that CS exposure changes components of saliva and salivary glands. Saliva is a body fluid that encounters exogenous materials and gases such as CS that penetrate the body. Free radicals, reactive oxygen species, reactive nitrogen species, carcinogens and, various toxic substances in inhaled CS have been suggested to mount a constant and direct attack on oral epithelial cells, gradually accumulating and causing a stepwise malignant transformation. It is known that sidestream smoke contains many more chemical compounds than mainstream smoke (Byrd, 1992). For instance, the concentration of carbon monoxide in sidestream smoke has been found to be 2.5-fold of that in mainstream smoke. Similarly, sidestream smoke contains 52-fold dimethylnitrosamine, 1.7-fold tar, 2.7-fold nicotine, 5.6-fold toluene, 16-fold naphthalene and 2.8-fold benzo(a)pyrene of the respective concentrations in mainstream smoke. Thus, the biological effect of sidestream smoke could be stronger than that of mainstream smoke.

Like human smokers (Gordon *et al*, 1975; Wack and Rodin, 1982), CS-exposed rats in this study consistently weighed less than the control rats, weighing 18.3% less by the end of 31 days of exposure. Passive smoking was shown by measuring the salivary cotinine concentration. It is clear that reduced body weight gain was caused by CS exposure. This could not be explained solely by a reduction in calories needed for weight maintenance as both groups of rats ate equivalent amounts of chow relative to their body weights. In human smokers, decreased weight has been attributed to possible alterations in calorie storage and/or an increase in metabolic rate (Wack and Rodin, 1982).

Although the level of nicotine as an indicator of passive smoking has a high degree of specificity, its biological half-life in blood is short (20–30 min), and as a result, the timing of sample collection after smoking is an important factor. As the biological half-life of cotinine, a nicotine metabolite, is much longer (30 h), the level of cotinine is a better indicator of passive smoking (Etter *et al*, 2000). In this study, using the

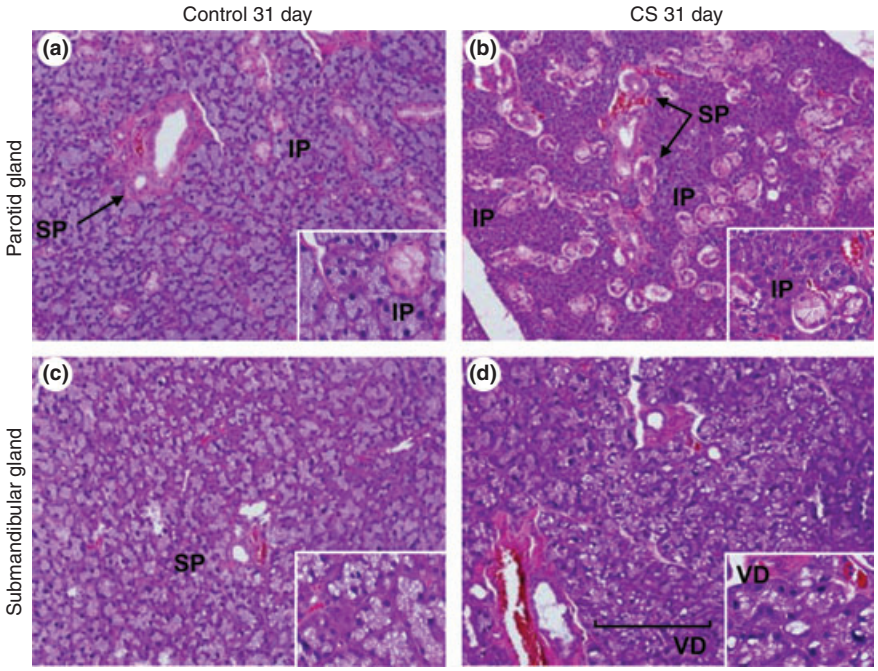

Figure 5 Histological changes in the salivary glands. The parotid (**a**, **b**) and submandibular glands (**c**, **d**) were embedded in paraffin and cut into sections at the end of the 31-day experimental period. These sections were stained by hematoxylin and eosin (HE). The CS rats showed vasodilation in SGs and hyperemia (**b**, **d**). In addition, a number of vacuolar degeneration existed in the SGs of CS rats (**b**, **d**). The inset shows an expanded view of each SG slide. IP, intercalated duct portion, SP, striated duct portion, VD, Vacuolar degeneration

cotinine detection microplate EIA kit, a difference between the cotinine levels in CS-exposed rats and control rats was observed. The salivary cotinine level varied among the samples after smoking.

While this study found that salivary flow was not affected by CS exposure at any time, decreases in total protein amount, amylase activity and peroxidase activity in saliva of 15-day CS group were detected that potentially could affect salivary antibacterial activity and oral health. The peroxidase found in saliva is believed to be one of the most important antioxidant enzymes in the oral cavity. CS contains hydrogen cyanide, which is metabolized by the liver to thiocyanate (SCN^-). SCN^- is specifically sequestered from the plasma by the parotid gland and is secreted by this gland into the oral cavity. Other investigators have reported higher salivary SCN^- levels in smokers than in non-smokers (Haley *et al*, 1983; Jarvis *et al*, 1987). Salivary peroxidase, total protein and amylase levels were significantly decreased in 15-day CS group rats. It may be that salivary peroxidase, total protein and amylase levels gradually recovered at 30 days to counteract effects of CS exposure. Another possible explanation for the 30-day recovery of salivary proteins was that CS rats drank more water than the control rats.

In the CS-exposed rats, vasodilation and hyperemia in the parotid grand and submandibular gland were observed by histological analysis. Nicotine was shown to dilate the peripheral vessel (Argacha *et al*, 2008). It is known that nicotine causes severe morphologic and functional alterations in the salivary glands (Maier *et al*, 1988). In this study, the CS-exposed parotid gland had many IPs and SPs, and the central part of the submandibular gland had many VDs. Although there was hyperemia in the parotid grand and submandibular gland, inflammatory cells were not found infiltrating the salivary glands. Cigarette smoke exposure might not have been the cause of inflammation in the salivary glands. While tobacco is currently not classified as a salivary gland carcinogen, existing data regarding the relationship between smoking and salivary gland tumors are sparse (Sadetzki *et al*, 2008). In this study, there was no evidence that the inflammation of the salivary glands upon CS exposure led to carcinogenesis. This study suggests other studies examining the adverse impact of CS on salivary glands are warranted.

Acknowledgements

This work was supported by KAKENHI (21792161, Grant-in-Aid for Scientific Research (B)).

Author contributions

Y Fujinami designed the study, analyzed and interpreted the data, and wrote the manuscript. T Fukui analyzed the data. K Nakano analyzed and interpreted the histological data. T Ara interpreted the data and did the statistics analysis. Y Fujigaki analyzed cotinine data and helped to analyze the data. Y Imamura advised on the design of the study and helped to analyze the data. T Hattori advised on the technical aspects and helped to analyze the data. S Yanagisawa designed the study. T Kawakami designed the study and interpreted the histological data. PL Wang designed the study and worked on the critical revision of the manuscript.

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