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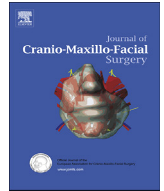

Inhibition of notch signaling pathway temporally postpones the cartilage degradation progress of temporomandibular joint arthritis in mice☆

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Purpose: The aim of this study is to explore the role of Notch signaling pathway in the initiation and progression of temporomandibular joint osteoarthritis (TMJOA).
Methods: 48 mice were divided into DAPT-TMJOA, Control-TMJOA and Control-Sham groups. Animals received discectomy/Sham surgery in their right TMJ, following the DAPT/saline intra-articular injections every week. Mice were sacrificed at 1/4/8 weeks post-surgery. Safranin-O and H&E staining were performed on the TMJ sections for the modified Mankin's score. qPCR and immunohistochemistry were used to evaluate Notch1, Jagged1 and Hes5 expressions.
Results: The mRNA expressions of Notch1, Jagged1 and Hes5 were significantly increased in Control-TMJOA group compared with Control-Sham group. Immunostaining revealed a dramatic elevation of Notch1, Jagged1 and Hes5 signals distributed in the cartilage at 1 and 4 weeks after discectomy. However, the increased number of those immuno-positive cells turned down at 8 weeks after surgery. DAPT treatment partially rescued the elevated mRNA expression and immuno-positive cell numbers of Notch1, Jagged1 and Hes5. More importantly, the cartilage destruction during TMJOA was delayed by DAPT treatment, analyzed by modified Mankin's score.
Conclusion: Notch signaling participates in the onset and development of TMJOA. Inhibiting Notch signaling activation by DAPT can partially delay the progress of TMJOA.
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1. Introduction

Osteoarthritis (OA) is a degenerative disease that affects all joint structures. OA is a heterogeneous syndrome with different clinical phenotypes more than a unique disease (Castaneda et al., 2014). When OA occurs in temporomandibular joint (TMJ), patients exhibited various symptoms, including jaw stiffness with pain, masticatory atonia, limit of mouth opening or progressively increasing anterior open bite (Blaney Davidson and van der Kraan, 2007; Shi et al., 2017). Although various methods, including open surgery and TMJ arthroscopy have been reported effective in reconstructing mandibular function and managing clinical symptoms in temporomandibular joint disorders patients, none of the current treatments yielded satisfactory outcomes in rescuing the degraded cartilage (Salash et al., 2016; Saeed and Kent, 2003). This is due to the heterogeneity of the manifestations and the unclear pathological mechanisms of OA. Under this circumstance, successfully rescuing or minimizing the progression of this disease is a formidable challenge (Leonardi et al., 2004; Zhang et al., 2011).

Notch signaling pathway plays a critical role in proliferation, differentiation, survival and apoptosis of cells as well as the formation and development of organs (Artavanis-Tsakonas et al, 1995, 1999; Lai, 2004; Brou, 2009). After binding to the specific ligands, Jagged and Delta, the Notch receptors are activated, giving rise to immigration of Notch intracellular domain (NICD) into the nucleus and binding to the cofactors CBF1/suppressor of hairless/Lag-1 (CSL), MAML-1 (mastermind-like-1) and p300/CBP(Mumm and Kopan, 2000; Mumm et al, 2000). The translocation of the Notch intracellular domain leads to the transcriptional activation of downstream target genes, which mainly are hairy enhancer of split

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Table 1
Primers for qPCR.

Gene	Forward Primer	Reverse Primer
Notch1	5′-GTCTCTACAGTGCTGGAAGT-3′	5′-CATACAGGGGGTTGCACTGT-3′
Jagged1	5′-CTGTATCTGTCCACCTGGCT-3′	5′-GGACACAGACACTGGAATCT-3′
Hes5	5′-GACCGCATCAACAGCAGCAT-3′	5′-GATGTCGGCCTTCTCCAG-3′
ACTB	5′-GAAGATCAAGATCATTGCTCCT-3′	5′-TACTCCTGCTTGCTGATCCACA-3′

(HES) and HES-related repressor (HERP), in which Hes1 and Hes5 count most in mammals (Ray et al., 1999; Strooper et al., 1999; Capell et al., 2000; Iso et al., 2003).

Previous studies showed that in the pathological process of osteoarthritis, the Notch signaling pathway can be blocked to repair damaged cartilage in knee OA (Karlsson et al., 2008). However, how Notch signaling correlates with the initiation and progression of TMJOA has not been well elaborated yet. In this study, we use N-[N-(3,5-diflurophenylacetate)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) to block the Notch signaling pathway to explore the mechanism and roles of Notch signaling that affect the onset and development of TMJOA.

2. Materials and Methods

2.1. Animals

Healthy adult female Kun Ming mice (33–35 g body weight, 8-week-old) were used for the experiments. Animals were fed with tap water and food for easy access under standard temperature and humidity conditions. Animals were randomly allocated into three groups: DAPT-TMJOA group, Control-TMJOA group, and Control-Sham group. Mice in DAPT-TMJOA group and the Control-TMJOA group received right TMJ discectomy to generate the TMJOA model as previously reported (Lan et al., 2017) (briefly described in 1.2). As a control, Sham surgery was performed in the Control-Sham group, in which group the condyles were exposed without further operation, and the wound was then sutured. 30 min after surgery and thereafter once per week, mice were respectively injected with γ-secretase enzyme blocking agent DAPT (dose = 50 mg/kg body weight, Stemcell Technologies, Vancouver, Canada) or normal saline using BD Micro-Fine 1 ml 29G syringe (BD Bioscience, Franklin Lakes, NJ) at similar volume as control (0.1 ml).

16 mice of each group were sacrificed respectively at 1 week, 4 weeks and 8 weeks after surgery. 6 mandibular condyles from the TMJOA/Sham surgical sites were used for histological examinations, and the other 10 condyles from the TMJOA/Sham surgical sites were used for quantitative polymerase chain reaction (qPCR) test.

All experimental protocols were approved by the Animal Ethics Committee o Sichuan University.

2.2. Surgical Procedure

Mice were anesthetized with 10% chloral hydrate through intraperitoneal injection (dose = 0.3 ml/100 g), and were submitted to local anesthesia by subcutaneous injection of 2% Lidocaine (dose = 0.05 ml/mouse). A “T” incision was made on the right TMJ region, then a blunt separation was made to separate submucosal tissue and muscle. After exposing the submandibular ramus, tissue was detached along the ramus until the capsule was seen. Then, the disc on the right TMJs were removed in DAPT-TMJOA and Control-TMJOA groups. The condyles of Control-Sham mice were only exposed without discectomy.

2.3. RNA extraction and RT-PCR

Total RNA was extracted from the cartilage region of the OA/control condyles using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was generated with Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, California, USA). Quantitative real-time PCR with SYBR was performed on the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, normalized with β-actin housekeeping gene expression, and presented as fold changes relative to the control. Sequences of primer pairs for qPCR were listed in Table 1.

2.4. Histologic Examination

Skulls were harvested and fixed in 4% paraformaldehyde for 48 h, and were decalcified in 15% ethylenediaminetetraacetic acid (EDTA) for 45 days prior to paraffin embedding process. After 3 weeks of decalcification, only the right skull was retained and continued to decalcification until paraffin embedding. 5 μm sections were cut and stained with hematoxylin-eosin (H&E) and Safranin-O. At each time point, 6 sections of each group were randomly selected, and were blindly scored by 3 independent observers using a modified Mankin's score system (Table 2).

2.5. Immunohistochemistry

At first, sections for immunostainings were treated with 5% bovine serum album (Goldbridge, Beijing, China) for 1 h at room temperature to prevent non-specific binding, and were immunolabelled with the following antibodies overnight at 4 °C: rabbit anti-mouse activated Notch1 (Ab52301, 1:100, Abcam, Cambridge, UK), rabbit anti-mouse Jagged1 (Ab7771, 1:200, Abcam, Cambridge, UK), rabbit anti-mouse Hes5 (Ab107593, 1:200, Abcam, Cambridge, UK). Sections were stained at room temperature for 2 h with goat anti-rabbit lgG secondary antibody (SP-9001, 1:1, Goldbridge, Beijing, China).

Table 2
Modified Mankin's score system used to evaluate cartilage pathology.

(1) Pericellular Safranin-O staining	
a. Normal	0
b. Slightly enhanced	1
c. Intensely enhanced	2
(2) Background Safranin-O staining	
a. Normal	0
b. Slightly increased or decreased	1
c. Severe increase or decrease	2
d. No staining	3
(3) Arrangement of chondrocytes	
a. Normal	0
b. Appearance of clustering	1
c. Hypocellularity	2
(4) Cartilage structure	
a. Normal	0
b. Fibrillation in superficial layer	1
c. Fibrillation beyond superficial layer	2
d. Missing articular cartilage	3

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