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# DNA DOUBLE-STRAND BREAK REPAIR IS ACTIVATED IN CAROTID ARTERY RESTENOSIS

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**Abstract**

Carotid stenosis is a narrowing or constriction of the inner surface of the carotid artery, usually caused by atherosclerosis. In the past decades, carotid angioplasty and stenting (CAS) has been developed into a credible option for the patients with carotid stenosis. However, restenosis remains a severe and unsolved issue after CAS treatment. Currently, the molecular mechanisms involved in the restenosis are still largely unclear. In this study, we found that the double-strand DNA breaks (DSBs) were induced by oxidative stress in the human carotid artery with restenosis by examining the level of γH2AX in the artery tissues. We further analyzed the activation of DNA damage repair pathways in the carotid restenosis. Our results suggest that non-homologues end joining (NHEJ), but not homologous recombination (HR), is mainly activated in the artery tissues with restenosis. Our results may provide clues to develop a new therapeutic strategy for carotid artery restenosis following CAS treatment.

***Key words:*** Carotid artery restenosis, Oxidative Stress, DNA damage, DNA damage repair.

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## INTRODUCTION

Carotid stenosis is a narrowing or constriction of the inner surface of the carotid artery (6, 31). It is a major risk factor for stroke that leads to brain damage (26, 35). Carotid stenosis is usually caused by atherosclerosis (34, 40), characterized by the atherosclerotic plagues accumulating in the artery wall, thus occluding the blood flow. In the past decade, carotid angioplasty and stenting (CAS) has been developed into a credible alternative treatment to carotid endarterectomy (CEA) for the patients with symptomatic moderate- and high-grade stenosis (16, 19). However, restenosis still remains an unsolved issue following CAS treatment, and the restenosis rate is even higher than that following CEA treatment (1, 13). Restenosis is the arterial wall's healing response to mechanical injury and comprises two major processes neointimal hyperplasia (i.e. smooth muscle migration/proliferation, extracellular matrix deposition) and vessel remodeling (14, 24). Thus, to develop more effective methods for preventing restenosis, a better understanding of the molecular mechanism of restenosis is important.

Emerging evidences have supported a crucial role of DNA damage response in the development and progression of atherosclerosis (10, 20). These findings support the concept that prolonged exposure to risk factors such as dyslipidemia, smoking and diabetes mellitus, which induces the accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS are critical hazards causing DNA damage within the atherosclerotic plaque (10, 20). Excessive DNA damage induces genomic instability at cellular level, which leads to cell cycle arrest, apoptosis, premature vascular senescence, and affects vascular functions (23, 39). Moreover, increased oxidative stress is considered as a major characteristic of restenosis following CAS or CEA treatment, which plays an important role in neointima formation (2, 41). It has been demonstrated that oxidative DNA adduct, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG), and DNA repair enzyme, PARP1, are quickly increased in carotid arteries following carotid angioplasty (18, 30) . These oxidative species mainly induce DNA single-strand breaks (SSBs) that are immediate repaired by base excision repair and nucleotide excision repair machinery (21, 38). However, extensive biological analyses of other tissues and diseases indicate that not all the SSBs in nucleus could be repaired (8, 33). Instead, SSBs could be converted into DNA double-strand breaks (DSBs), which is a much more deleterious type of DNA damage (8, 33). To date, it is not clear whether DSBs occurs during restenosis. Since DSBs activate a global DNA damage response for cell viability and proliferation (3, 15, 22), we examined DSB-induced DNA damage response during the formation of carotid restenosis and characterized the DSB repair pathways involved in this process using the human carotid artery samples excised from the patient with restenosis following CAS treatment. Our study reveals an important biological process that is activated in carotid artery restenosis. It might be useful for future clinical treatment of carotid artery restenosis.

## MATERIALS AND METHODS

### *Carotid artery tissues*

Three carotid artery tissues were surgically removed from the patients with carotid restenosis following CAS treatment at Xuan Wu Hospital in China. The human artery tissues used in this study were harvested after obtaining approval from the ethics committees at Capital Medical University and University of Michigan and from the patients who gave written informed consent.

### *Antibodies*

Mouse anti-β-actin monoclonal antibody (AC-15) was purchased from Sigma. Mouse anti-γH2AX monoclonal antibody (JBW301) was purchased from Upstate. Rabbit anti-Ku70, anti-Ku80 and anti-RAD51 polyclonal antibodies were purchased from Cell Signaling. Rabbit anti-H2AX polyclonal antibody and rabbit anti-Ligase IV polyclonal antibody were purchased from Novus.

### *Histopathology*

The artery specimens were fixed in 4 % paraformaldehyde for 24 hr, dehydrated, and embedded into paraffin wax, then sectioned to 4 μm slides and processed for H&E staining.

### *Immunofluorescent staining*

To perform immunofluorescent staining for human γH2AX, Ku70 and RAD51, sections were deparaffinized and washed in PBS. After blocked by rabbit serum for 10 min at room temperature, the sections were incubated for 1 hr with mouse anti- γH2AX (1:500), rabbit anti-Ku70 (1:500) and anti-RAD51 (1:500) respectively. The slides were then washed in PBS and incubated with FITC-coupled secondary antibodies (1:1000, Jackson ImmunoResearch Laboratories) for 30 min at room temperature and counter-stained with 1 ml of 5 μg/ml Hoechst 33342 (Sigma) for 2 min. The sections were washed again three times with PBS and then visualized by a fluorescence microscope.

### *Protein extraction and Western blotting*

Protein samples were extracted from the tissue using total protein extraction kit (Millipore, # 2140). The protein concentration was determined using a BCA standard curve. Equal amounts of protein extract were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked in TBST containing 5% milk at room temperature for 1 hr. After washing with TBST, the blocked membranes were probed with indicated primary antibodies overnight at 4 °C. After three consecutive 10-min washes with TBST, the membrane was incubated with HRP-conjugated goat-anti-rabbit or goat-anti-mouse secondary antibody for 1 hr. The membrane was washed again three times with TBST and developed using the ECL+detection system (GE Healthcare).

### *qRT-PCR*

Total RNA was isolated from the carotid artery tissue using Trizol (Invitrogen) according to the manufacturer's instruction. Approximately 500 ng of total RNA was reverse transcribed using oligo-dT primers. The cDNA was utilized as a template to amplify target genes with SYBR Green PCR Master Mix (Invitrogen). Specific primers were listed in Table 1. Each RNA sample was evaluated in triplicate. Gene expression results were analyzed with the ΔΔCt method and normalized to β-actin expression. The qRT-PCR assay was performed on Bio-Rad iQ5 instrument. The data were analyzed using Optical System Software 2.0.

## RESULTS

In the past decade, CAS has been proposed as a safe and effective alternative to CEA for patients with carotid stenosis (16, 19). However, restenosis remains a severe and unsolved issue following CAS treatment (1, 13). One option for these patients is to surgically remove the blocked part of the carotid artery together with stent, and use graft to replace the removed vessels (25). We harvested the carotid artery samples following this type of surgery at Xuan Wu Hospital in China. H&E staining was performed and neointima hyperplasia was observed in the carotid artery with stenosis, but not in the adjacent normal artery tissue (Fig. 1).

Previous studies have demonstrated that oxidative stress-induced DNA damage occurs in the neointima of carotid artery with restenosis after angioplasty, characterized by the significant increase of oxidative DNA adduct, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) (41). Since ROS products can further result in SSBs and DSBs (9, 21, 38), we asked whether DSBs could be observed in neointima of carotid artery with restenosis. We examined γH2AX, a surrogate DSB marker by immunofluoresent staining (29). We found that positive γH2AX staining existed in neointima of carotid artery with restenosis, but not in the adjacent normal carotid artery (Fig. 2A). Western blotting compared the level of γH2AX in the carotid artery tissue with restenosis from three patients. Adjacent normal artery was used as the control. The results showed that γH2AX increased in all three artery tissues with resteno-

**Table 1.** qRT-PCR primers

| Gene | Direction | Sequence (5'-3') |
|---|---|---|
| Ku70 | Sense | TACAGAGATATCATCAGCATAGCAG |
| | anti-sense | CACTATATCTTTGTTGAGCTTCAGC |
| Ku80 | Sense | TGAGTGTTTAGTGTATGTGCAGCTG |
| | anti-sense | CATCAACAGCATTCAACTGTGCCTC |
| Ligase IV | Sense | ATAACTACACTGATCAGTTTGGTGC |
| | anti-sense | TGTATTAGGATTATAGGCCATCATC |
| RAD51 | Sense | AGAATTCCGAACTGGGAAGACCCAG |
| | anti-sense | ACAGATCTGGGTCTTCCCAGTTCGG |
| β-actin | Sense | ACTGTGCCCATCTACGAGGGGTATG |
| | anti-sense | CGTAGCACAGCTTCTCCTTAATGTC |

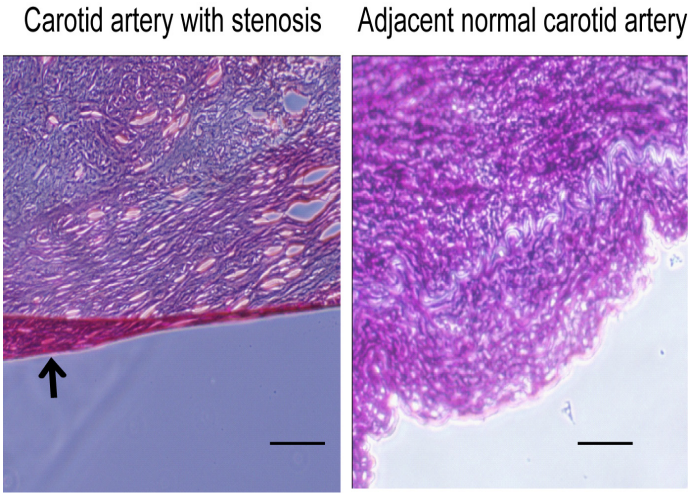


**Figure 1. Neointima hyperplasia in the carotid artery tissue with restenosis.** Carotid artery with restenosis was surgically removed from the patients and harvested for H&E staining. Thickened neointima was observed (dark arrow). Adjacent normal carotid artery was shown as the control. Scale bars: 200 μm.

sis in different degree, but no γH2AX was detected in the normal artery tissue (Fig. 2B). These results are consistent with our immunofluorescent staining result. Thus, our study suggests that DSBs occurred in the carotid artery with restenosis, especially in the thickened neointima area.

In response to DNA damage, a variety of DNA damage response such as different DNA repair pathways is immediately activated. For DSBs, usually two major DNA repair pathways are activated to repair these lesions. One is homologous recombination (HR) for the slow and error-free repair of DSB; the other is non-homologous end-joining (NHEJ) for the simple and fast repair of DSB (7, 11). The major effectors of NHEJ pathway include Ku70, Ku80, DNA-PKcs, Artemis, Ligase IV and XRCC4. Ku70, Ku80 and DNA-PKcs recognize the DNA lesions and load Artemis for the DNA end processing, while Ligase IV and XRCC4 ligate the ends of DSBs (11, 28). To determine the role of NHEJ pathway during the DNA damage repair process in the carotid artery with restenosis, we performed immunofluorescent staining to detect the expression level of Ku70, the key DNA lesion sensor for the activation of

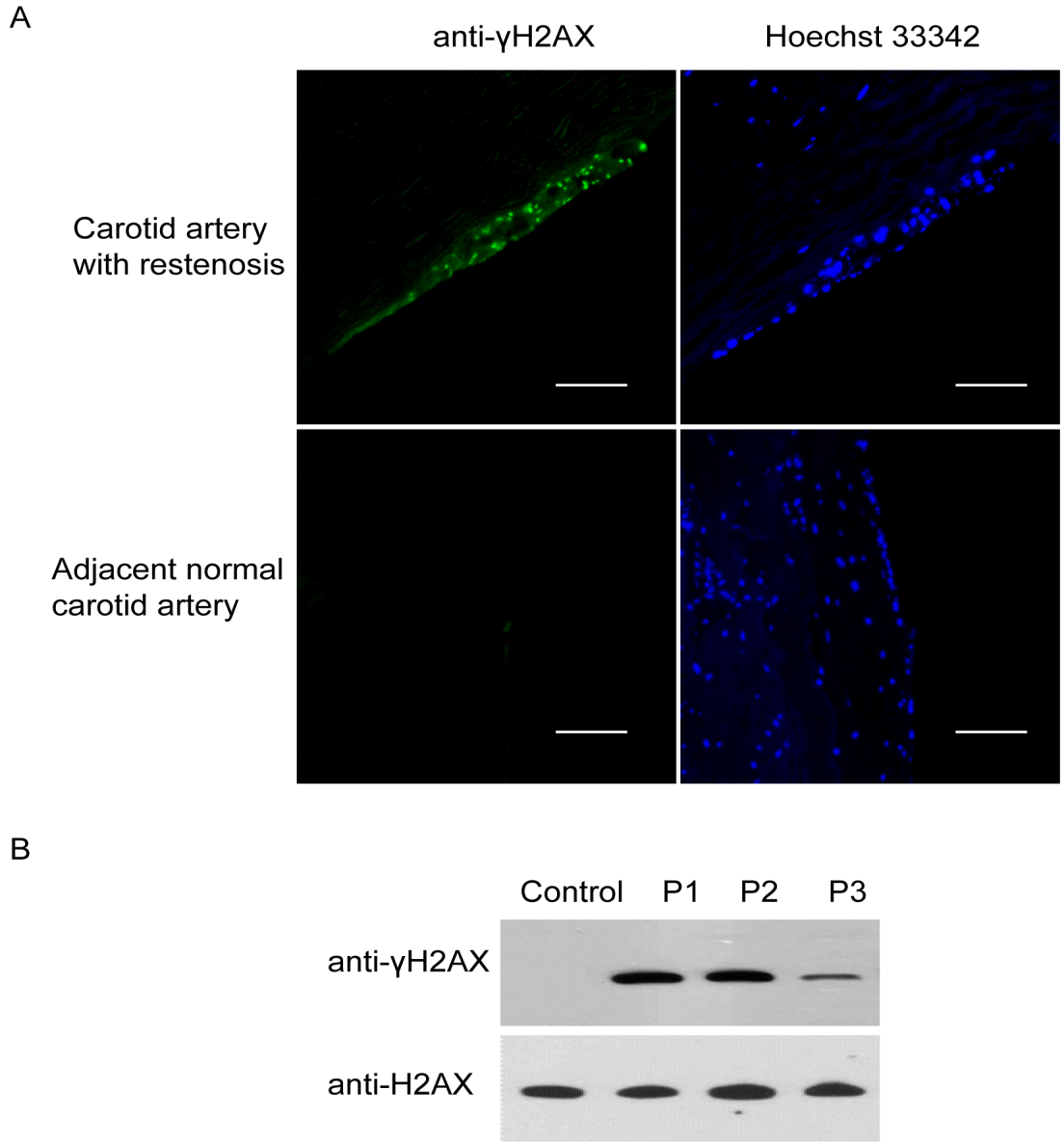


**Figure 2. DSBs occur in carotid artery with restenosis.** (A) Immunofluorescent staining was performed to detect the level of γH2AX in the carotid artery tissues. Significantly positive γH2AX staining was observed in the neointima area of carotid artery with restenosis, but not in the normal carotid artery. Scale bars: 200 μm (B) Western blotting result shows the increased γH2AX in the carotid artery with restenosis. The adjacent normal artery was used as the control. H2AX protein level was shown as loading control. P1, P2 and P3 represent the tissue from different patients, and control represents the adjacent normal artery.

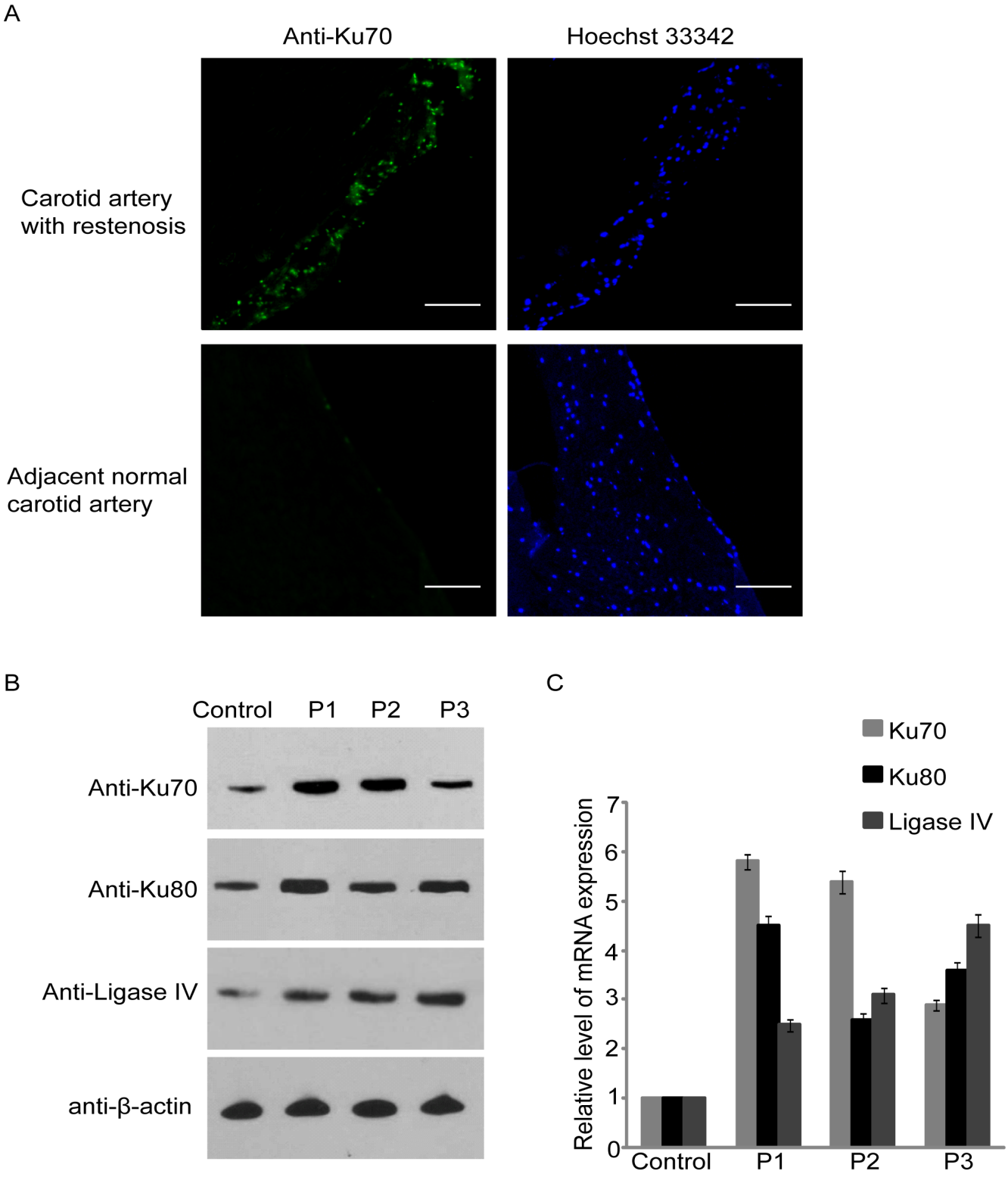


**Figure 3. NHEJ pathway participates in DSB repair in the carotid artery with restenosis.** (A) Immunofluorescent staining was performed to examine the Ku70 expression level in the carotid artery tissues. Significant increase of Ku70 level was observed in the carotid artery with restenosis. The adjacent normal artery was used as the control. Scale bars: 200 μm (B-C) Western blotting and qRT-PCR were used to detect the changes of the expression of NHEJ-related gene products. Ku70, Ku80 and Ligase IV expression levels were significantly increased in the carotid artery tissue with restenosis than those in the normal artery. Error bars indicate s.d. (n=3).

NHEJ repair (17, 28). Significant increase of Ku70 expression level was observed in the neointima area, compared with adjacent normal carotid artery (Fig. 3A). Moreover, with Western blotting and qRT-PCR, we further examined the expression levels of Ku70, Ku80 and Ligase IV. The results showed that all these three NHEJ pathway-related gene products elevated in different degree in the artery tissue with restenosis compared with that in normal artery (Fig. 3B, C). In particular, Ligase IV is the final effector for NHEJ repair (28, 36). Elevation of Ligase IV suggests that NHEJ pathway is activated during carotid artery restenosis. Taken together, our results indicate that NHEJ pathway plays an important role for DNA damage repair in the carotid artery with restenosis.

Besides NHEJ, HR is another type of DSB repair (11, 27). It has been shown that RAD51 is the key effector in HR repair (4, 11). Thus, we next examined the expression level of RAD51 in the carotid artery tissue with restenosis.

However, no significantly positive staining of RAD51 was observed in the carotid artery tissue (Fig. 4A). Western blotting and qRT-PCR were performed and further confirmed that no significant change of the expression level of RAD51 was observed in the artery with restenosis (Fig. 4B, C). Thus, our study suggests that NHEJ pathway may play an important role to repair DSBs in the carotid tissue with restenosis.

## DISCUSSION

Restenosis following CAS is a complex process (1, 13). Although the cellular events involved in restenosis are evident, the molecular mechanisms responsible for restenosis are unclear. More and more evidences suggest that oxidative stress plays an important role in restenosis (2, 41), and the level of oxidative DNA damage marker, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) significantly increases in the neointima of artery with restenosis (41). Here, our study suggests that DSBs are also induced in the carotid restenosis. And we demonstrate that NHEJ, but might not HR, plays an important role for the DNA damage repair in the artery with restenosis.

This study is likely beneficial for developing new prevention strategy. For the patient with carotid artery stenosis, it is a great challenge to prevent restenosis following CSA or CEA treatment (1, 13). Although some prevention strategies have been tried, such as using drug-eluting stents (32, 37) and administering anti-platelet drugs immediately after surgery (12), the effects are still not satisfactory. Activation of NHEJ pathway ensures the cell viability and facilitates cell proliferation (3). Thus NHEJ repair could be a leading factor for artery restenosis. Additionally, carotid artery restenosis is characterized by neointima hyperplasia (14, 24). And decrease in apoptosis contributes to neointima hyperplasia by prolonging the life span of intimal cells (5). Inhibition of NHEJ is likely to accumulate DSBs, which induces apoptosis. Thus, inhibition for NHEJ pathway may be a new approach for the suppression of neointima hyperplasia following CAS treatment.

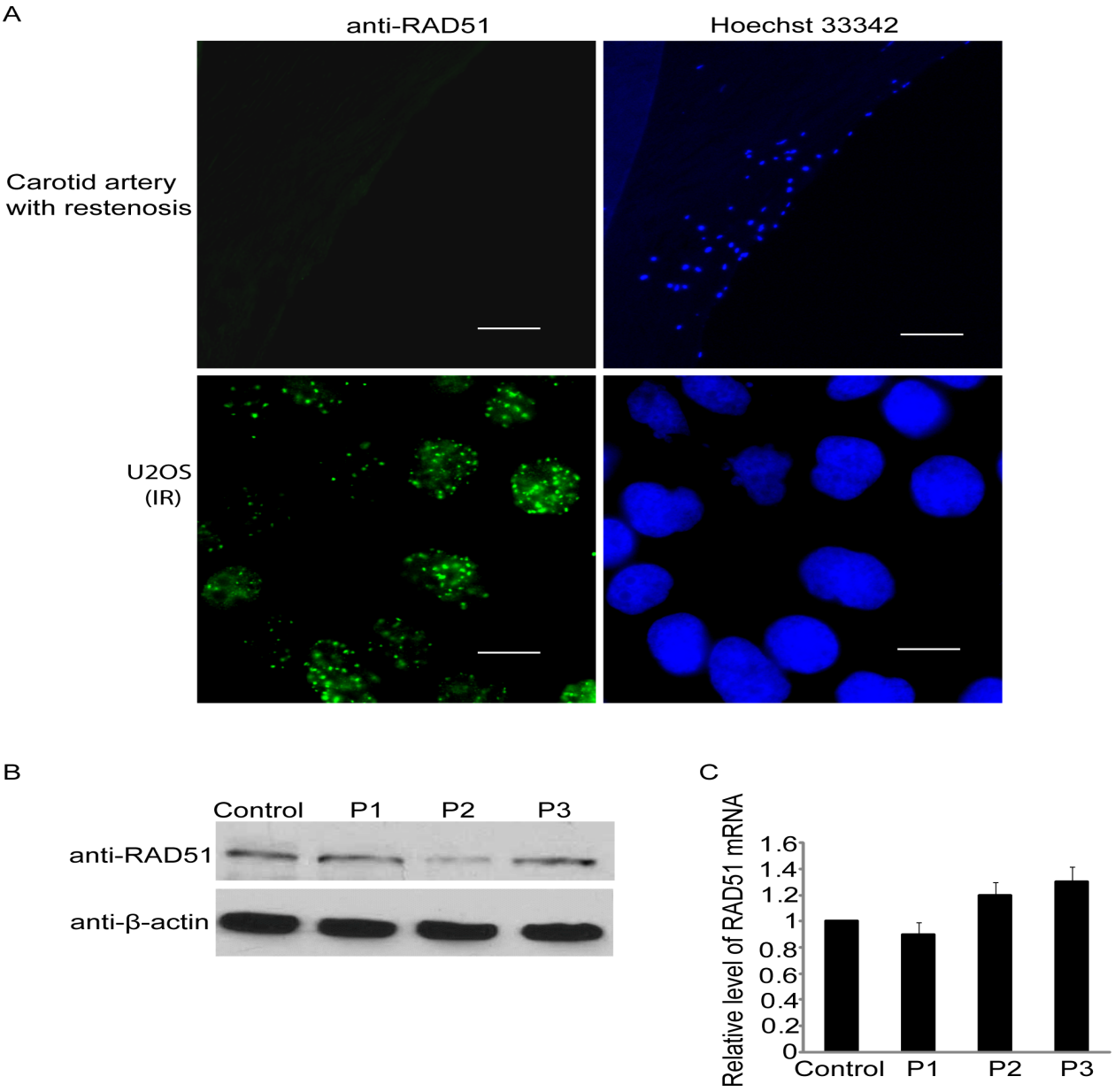


**Figure 4. The expression of RAD51 does not change in the carotid artery with restenosis.** (A) Significantly positive RAD51 immunofluorescent staining was not observed in the carotid artery tissue with restenosis. Immunofluorescent staining of RAD51 in U2OS cells exposed to ionizing radiation (5 Gy) was shown as positive control. Scale bars: 200 µm, artery tissues (Upper panel); 20 µm, U2OS cells (Lower panel) (B, C) Western blotting and qRT-PCR were used to detect the expression level of RAD51 in the carotid artery with restenosis and normal carotid artery. No significant change was observed. Error bars indicate s.d. (n=3).

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