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# Radiotherapy inhibits neointimal hyperplasia after artificial vascular replacement through Skp2/P27kip1

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## ABSTRACT

We aimed to establish an animal model of abdominal aortic vascular replacement in mongrel dogs to investigate the effect of extracorporeal radiotherapy on the intima. Twenty healthy mongrel dogs were randomly divided into four groups: 5-week control group, 5-week radiotherapy group, 10-week control group and 10-week radiotherapy group. We first performed an artificial vascular replacement of the abdominal aortic segment. The radiotherapy group received external radiotherapy with a dose of 7 Gy for 4 days. The thickness of neointimal hyperplasia, immunoreactivity and expression of proliferation-related factors were detected by hematoxylin and eosin (HE) staining, immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR )and western blotting at 5 and 10 weeks after the reconstruction. The results showed that the intimal thickness of the artificial blood vessel in the 5- and 10-week radiotherapy groups was thinner than that in the control groups by HE staining. The immunoreactivity and expression levels of Skp2, c-Myc and CyclinE1 were significantly decreased in the radiotherapy groups than those in control groups by immunohistochemistry, qRT-PCR and western blotting. On the contrary, immunoreactivity and expression levels of $P27^{kip1}$ were increased. In conclusion, we discovered that postoperative external radiotherapy significantly decreases the intimal hyperplasia of artificial blood vessels by regulating c-Myc-Skp2-P27-CyclinE1 network.

**Keywords:** radiotherapy; artificial vascular; neontimal hyperplasia; Skp2; P27

## INTRODUCTION

In recent years, vascular reconstruction has been used more frequently in clinical practice [1]. The materials of vascular reconstruction include autologous blood vessels and artificial blood vessels [2]. However, due to the limitations of autologous blood vessels, artificial blood vessels have been widely used. Artificial blood vessels are mostly made of synthetic materials, such as nylon, Dacron and polytetrafluoroethylene (PTFE), and are substitutes for many severely stenotic or occlusive blood vessels [3]. For example, when a patient with kidney disease has an arterial occlusion after hemodialysis, the artificial blood vessels can be used as a shunt [4]. In liver surgery, artificial blood vessels have been successfully applied to vein reconstruction [5]. Artificial blood vessels have also been reported to be used in vascular reconstruction after tumor resection, which greatly improves the resection rate of tumors and ensures the blood supply of important organs [6, 7]. Therefore, artificial vascular replacement is one of the effective methods to treat peripheral vascular diseases and revascularization after tumor resection.

Artificial vascular replacement has brought great hope to patients, but neointimal hyperplasia of proximal and distal anastomosis is still the main cause of medium (3–24 months) and long-term transplantation failure (more than 2 years) [8, 9]. The endothelial hyperplasia can cause vascular anastomotic stenosis, and any endothelial injury caused by internal operation of endothelium can cause an abnormal proliferative reaction of the intima [10].

Radiotherapy has been used in the clinical treatment of malignant tumors for decades, which improves the cure rate of tumors [11]. In animal models or clinical studies, radiotherapy inhibits intimal

hyperplasia caused by balloon injury and angiogenesis. The formation of intimal hyperplasia also involves many cellular and molecular mechanisms, such as platelet activation, leukocyte recruitment, coagulation cascade activation, migration and proliferation of smooth muscle cells (SMC) [12–15]. However, the specific mechanism of the inhibitory effect of extracorporeal radiotherapy on intimal hyperplasia of artificial vascular anastomosis needs further investigation.

In this study, we established an animal model of abdominal aortic vascular replacement for mongrel dogs to investigate the effects of postoperative extracorporeal radiation therapy on the vascular intima and explore its possible mechanism.

## MATERIALS AND METHODS

### Animals

A total of 24 mongrel dogs were purchased from the Animal Center of the Second Xiangya Hospital of Central South University (Changsha, China) weighing ($12 \pm 0.6$) kg, with food and water freely available. All dogs needed to be adapted to laboratory conditions at least 7 days before surgery and received expanded polytetrafluoroethylene (ePTFE) vascular grafts from the inferior abdominal aorta. Four dogs died after surgery, and the surviving mongrel dogs (20) were randomly divided into four groups: 5-week control group (n = 5), 5-week radiotherapy group (n = 5), 10-week control group (n = 5) and 10-week radiotherapy group (n = 5). All designs and animal procedures were approved by the Animal Care and Use Committee of Second Xiangya Hospital of Central South University.

### Surgical procedure

The dogs receiving intubation and mechanical ventilation were anesthetized using 3% pentobarbital sodium (30 mg/kg, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), and the inferior abdominal aorta was exposed by median laparotomy and retroperitoneal opening. After intravenous injection of 0.5 mg/kg heparin (Solarbio, Beijing, China) for 5 min, the proximal and distal inferior abdominal aorta was blocked by non-invasive vascular clamp for 30–40 min and replaced by ePTFE graft (Lifespan, Providence, USA) (Supplemental Fig. 1).

### Irradiation procedure

Ten dogs were selected for radiotherapy two weeks after surgery. Radiotherapy was given daily by a linear accelerator at a dose of 7 Gy for 4 days with a total dose of 28 Gy. The radiant area is approximately $3 \times 4$ cm$^2$ including two the anastomotic stomata. The control group was not irradiated.

### Histopathology examination

The samples were taken under terminal anesthesia after 5 and 10 weeks and cut into three sections: the proximal anastomosis, the distal anastomosis and the middle section of the graft. The proximal and distal samples were fixed in 10% paraformaldehyde (PFA; Solarbio, Beijing, China). The middle samples were stored in liquid nitrogen for quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting. The two anastomoses were longitudinally sectioned and embedded in paraffin, and the paraffin sections were stained with hematoxylin and eosin (HE; Solarbio, Beijing, China). Image-Pro plus 7.0 software (Media Cybernetics Inc., Bethesda, USA) was used to measure the thickness of intimal hyperplasia after staining.

### Immunohistochemistry

Proliferation-related factors, such as S-phase kinase-associated protein 2 (Skp2), P27$^{kip1}$(P27), c-Myc and CyclinE1, were detected by Histostain-Plus Kit (Thermo Fisher Scientific, Waltham, USA). Relevant experimental procedures were conducted in accordance with the instructions.

### Western blotting

The middle section of the graft was lysed in Radio Immunoprecipitation Assay (RIPA) buffer (Beyotime, Shanghai, China). BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) was used to test the concentration of proteins. Total proteins (20 $\mu$g) were subjected to 6–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to the polyvinylidene fluoride (PVDF) membranes. The membranes were cultured in 5% bovine serum albumin (BSA; Solarbio, Beijing, China) solution for 1 h and sequentially incubated with the primary antibodies overnight at 4°C, such as Skp2 (#4358), P27 (#3686), c-Myc (#5605), CyclinE1 (#20808) and GAPDH (#8884, all from Cell Signaling Technology, Danvers, USA). The membranes were washed three times with TBST for 5 min each time, and then the HRP-labelled secondary antibodies (ab191866, 1 mg/ml, Abcam, Cambridge, USA) were added and incubated for 1 h at room temperature. Specific signals were detected using the ECL western blotting detection system.

### qRT-PCR

The RNAprep pure Tissue Kit (DP431; Tiangen, Beijing, China) was used to extract RNA. After grinding the middle section of the graft (20–30 mg) in 300 $\mu$l of lysis buffer, centrifugation was conducted in a freezing centrifuge at a speed of 12 000 rpm for 5 min. The supernatant was transferred to a new test tube and 80 $\mu$l of DNase I (Thermo Fisher Scientific, Waltham, USA) was added to remove the DNA contamination. Total RNA (1 $\mu$g) was reverse transcribed in a 20 $\mu$l solution containing random primers, Superscript IV RT, reaction buffer, dNTP, DTT and RNasin (Thermo Fisher Scientific, Waltham, USA). The sequences for specific primers were listed in Table 1. QRT-PCR was performed using ABI Prism 7900 instrument (Applied Biosystems, Waltham, USA). The expression level was normalized to GAPDH mRNA level, and analyzed by the comparative Ct method.

### Statistical analysis

The data were expressed as means ± standard deviation (SD) and analyzed by SPSS 17.0 statistical software (SPSS Inc., Chicago, USA). Student's *t*-test was used for statistical comparisons between the two groups. One-way ANOVA was used for the comparisons among several groups after the least-significant-difference test. $P < 0.05$ was considered to have statistical significance.

## RESULTS

### Radiotherapy suppressed neointimal hyperplasia in the graft

To evaluate the effect of radiotherapy on neointimal hyperplasia of grafts, we used HE staining to detect the proximal and distal anastomotic sites. The intimal thickness in the 5-week radiotherapy group

**Table 1. The primers used for qRT-PCR**

| Gene | Primers | Sequence(5′-3′) |
|---|---|---|
| GAPDH | Forward | CCATCTTCCAGGAGCGAGAT |
| | Reverse | TCACGCCCATCACAAACATG |
| Skp2 | Forward | TCCAGACCAGAGTAGCAACG |
| | Reverse | GCCTGCGGACTATGACAAAG |
| p27 | Forward | AAACGTGCGAGTGTCTAACG |
| | Reverse | CTCCTGCCACTCGTATTTGC |
| CyclinE1 | Forward | GCAGGGAGCGGGATGCGAAG |
| | Reverse | AGCGGGGAGCCTCTGGATGG |
| c-Myc | Forward | CCAGCAGCGACTCTAAGG |
| | Reverse | CCAAGACGTTGTGTGTTC |

(proximal: 230.09 ± 25.22 and distal: 233.34 ± 46.42) was thinner than that in the control group (proximal: 482.57 ± 73.09 and distal: 507.53 ± 62.92; $P < 0.05$, Fig. 1). Similarly, the intimal thickness in the 10-week radiotherapy group (proximal: 305.67 ± 35.00 and distal: 254.17 ± 52.68) was also thinner than that in the 10-week control group (proximal: 615.13 ± 89.03 and distal: 626.18 ± 78.02; $P < 0.05$, Fig. 1).

## Radiotherapy decreased the immunoreactivity of Skp2, c-Myc, CyclinE1 and increased the immunoreactivity of P27

In order to investigate the possible mechanism of radiotherapy for neointimal hyperplasia, we examined the immunoreactivity of several proliferation-related factors. As shown in Fig. 2, the immunoreactivity of Skp2, c-Myc and CyclinE1 in the 5- and 10-week radiotherapy groups were significantly decreased compared with their control groups ($P < 0.05$). On the contrary, the P27 immunoreactivity was increased after treatment with radiotherapy ($P < 0.05$, Fig. 2).

## Radiotherapy downregulated Spk2, c-Myc, CyclinE1 expression and up-regulated P27 expression

Finally, we used qRT-PCR and western blotting to examine the mRNA and protein expression of proliferation-related factors for further study the effect of radiotherapy on intimal hyperplasia. We found that the mRNA and protein level of Skp2, c-Myc and CyclinE1 in the 5- and 10-week radiotherapy groups were lower than that in their corresponding control groups ($P < 0.05$, Fig. 3 and 4). However, radiotherapy upregulated P27 mRNA and protein level in the 5- and 10-week radiotherapy groups compared with the control groups ($P < 0.05$, Figs 3 and 4).

## DISCUSSION

The complications often occur after vascular reconstruction, and intimal hyperplasia is one of the most important complications, which is also the main reason transplant surgery fails [16]. The researchers have discovered various therapies that inhibit or reduce neointimal hyperplasia, pharmacological interventions, lipid-lowering therapy and external beam and intraluminal radiation [17–20]. In this study, a mongrel dog artificial blood vessel replacement model was established to study the effect and mechanism of radiotherapy on neointimal hyperplasia. Meanwhile, our speculation about the possible mechanism of radiotherapy for neointimal hyperplasia was shown in Fig. 5.

A study found that the common pathway of cell proliferation is cell cycle, which is regulated and controlled by cell cycle regulatory system. The ubiquitin protease system is the main pathway for intracellular protein degradation [21]. Skp2 is a part of the Skp1-Cullin1-F-box (SCF) E3 ubiquitin-ligase complex, which regulates cell proliferation by regulating cell cycle regulators for ubiquitination. P27 is a recognized tumor suppressor gene [22]. The imbalance between Skp2 and P27 can affect a variety of cancers, such as colorectal, breast and gastric [23–25]. The inactivated Skp2 gene leads to the accumulation of P27, thus stopping the cell cycle of the G1 phase and blocking cell proliferation [26]. Caraballo *et al.* have also shown that c-Myc counteracts P27 effects, and low P27 level is usually associated with high c-Myc expression in human cancers. In addition, c-Myc induces P27 degradation through up-regulation of Skp2 expression in chronic lymphocytic leukemia (CLL) cells, suggesting that c-Myc is one of the upstream regulators of Skp2 and P27 [27]. Salon *et al.* reported that Skp2 and E2F-1 are negatively regulated in high-grade neuroendocrine lung tumors and stimulate their transcriptional activity on the cyclin E promoter [28]. A poor prognosis in breast cancer may result in high expression levels of Cyclin E and Skp2 as well as low expression level of P27 [28]. These studies indicate that c-Myc-Skp2-P27-cyclinE network is associated to the tumor cell proliferation.

In our study, we performed artificial vascular replacement surgery for the model dogs. After 5 weeks, a complete intimal hyperplasia was formed at the anastomotic end of the graft. The thickness of intimal hyperplasia of proximal and distal anastomosis after radiotherapy was thinned, indicating that the neointimal hyperplasia of the artificial blood vessels in the radiotherapy groups was significantly inhibited. It has been reported that SMC proliferation is stimulated in vein graft disease to cause intimal hyperplasia, and Skp2 and P27 networks can regulate SMC migration and proliferation [12–15]. Therefore, in order to further study the mechanism of neointimal hyperplasia in radiotherapy, we examined the immunoreactivity and expression levels of Skp2 and P27, and found that Skp2 in 5 and 10 week radiotherapy groups were lower than that in the

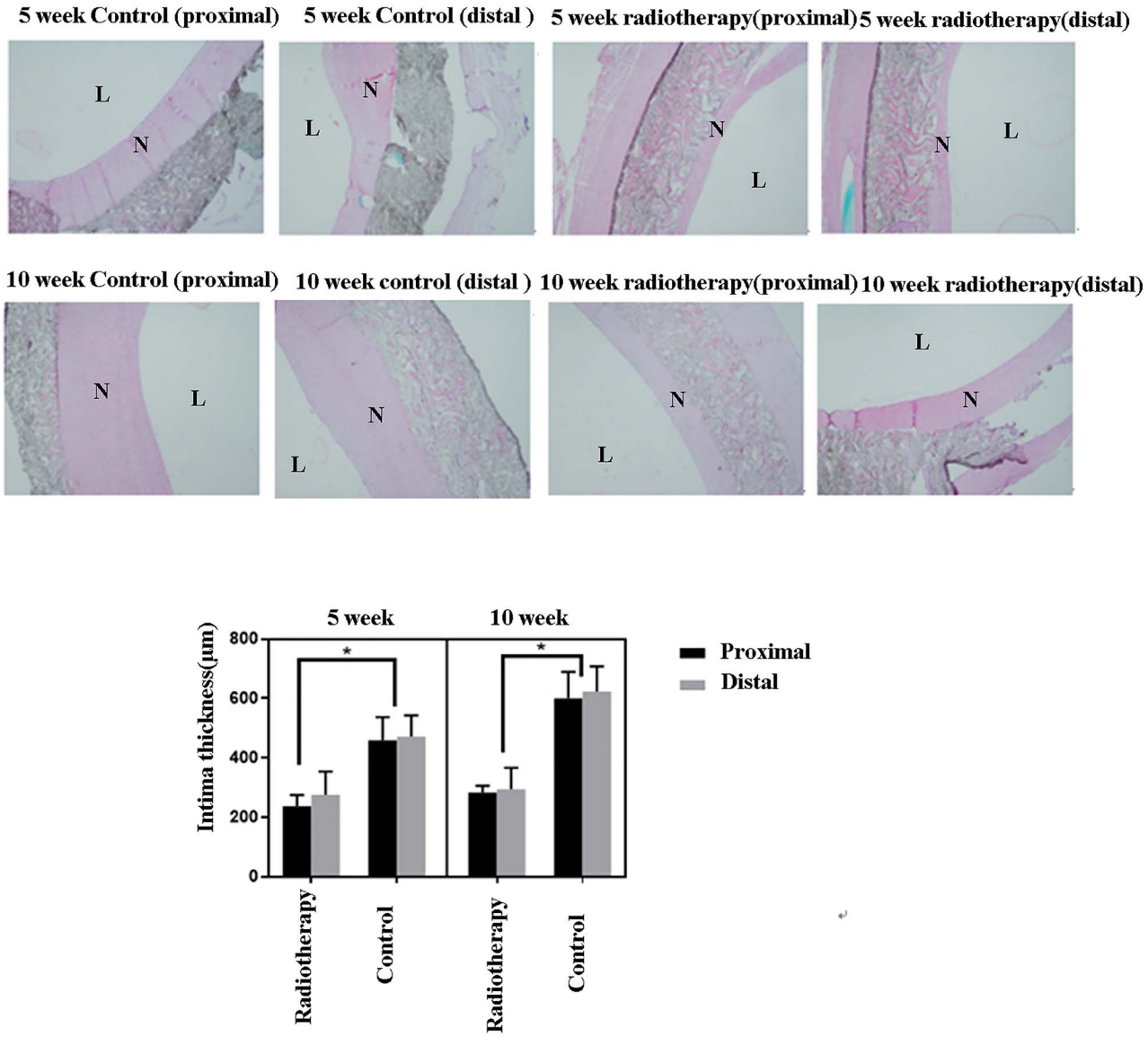


**Fig. 1. The effect of radiotherapy on the thickness of the graft neointima. Histological sections of the ePTFE graft were stained using HE. The thickness of the graft neointima was measured using image-analysis software in the proximal and distal regions of the graft. N: neointimal; L: vessel lumen.** * **$P < 0.05$.**

control groups. In contrast, P27 has increased immunoreactivity and expression level. Further, we also examined the expression of c-Myc and CyclinE1, and found that these results were similar to those of Skp2, that is, the expression of c-Myc and cyclinE1 in the 5 and 10 week radiotherapy groups were lower than those in the control groups. These results illustrate that c-Myc-Skp2-P27-CyclinE1 network is also involved in neointimal hyperplasia, and radiotherapy inhibits neointimal hyperplasia by inhibiting the network. Radiotherapy was performed according to our previous study. The source of radiotherapy is medical electron linac: Elekta precise5745 (Sweden). X-ray energy: 6MV. The irradiation area includes the whole process of artificial blood vessels and the autogenous abdominal aorta near the close and far end anastomosis. The irradiation area is determined as $3 \times 7$ cm$^2$, the total dose is 28Gy and it is irradiated four times every other day, the irradiation time is 150 seconds–200 seconds.

There were some limitations in this study. The cell death damage around the exposure area was not evaluated in this study. Other doses of

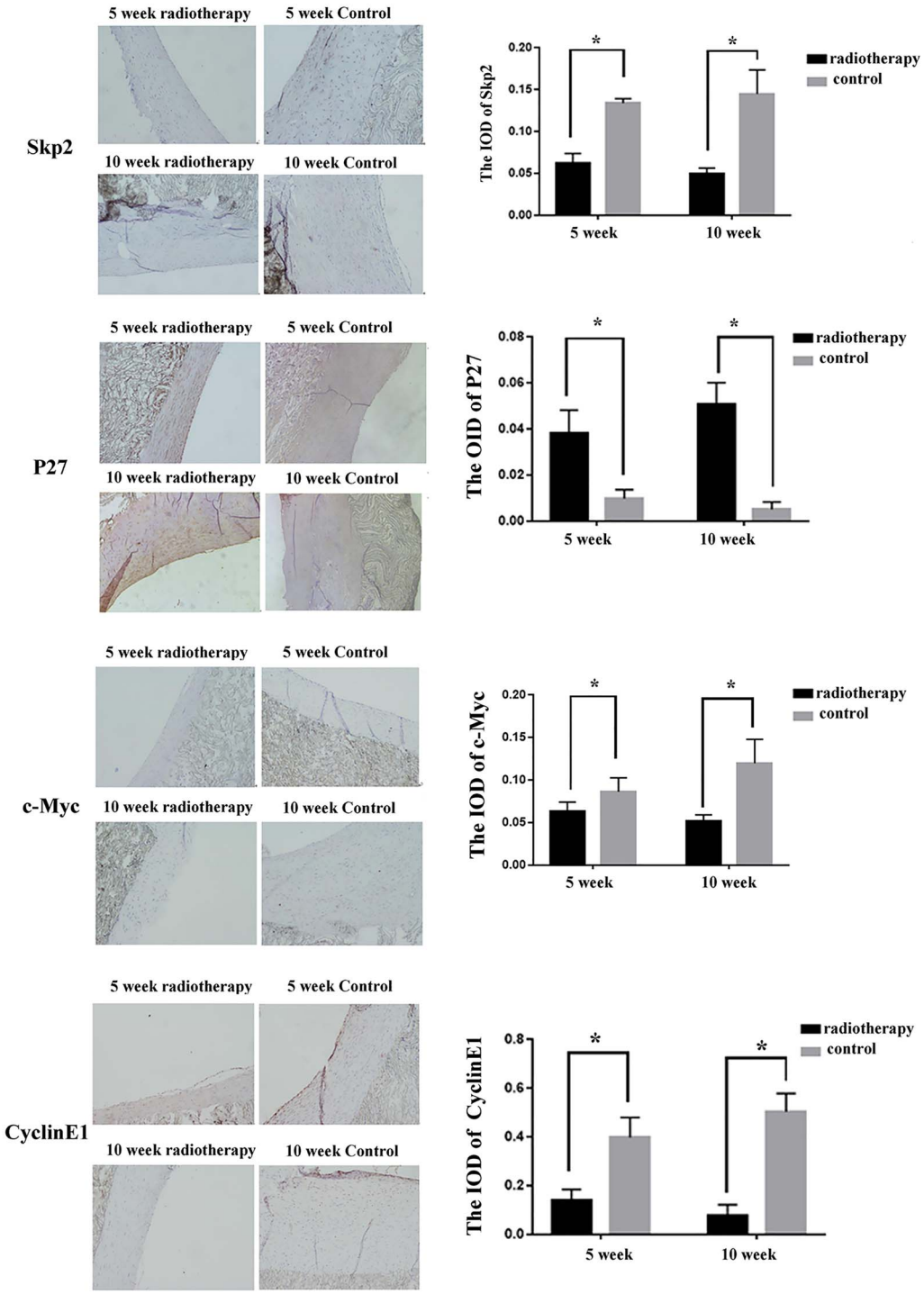


**Fig. 2. The effect of radiotherapy on immunoreactivity of proliferation-related factors. The immunohistochemistry was used to detect the expression of Skp2, P27, c-Myc and CyclinE1.** * $P < 0.05$.

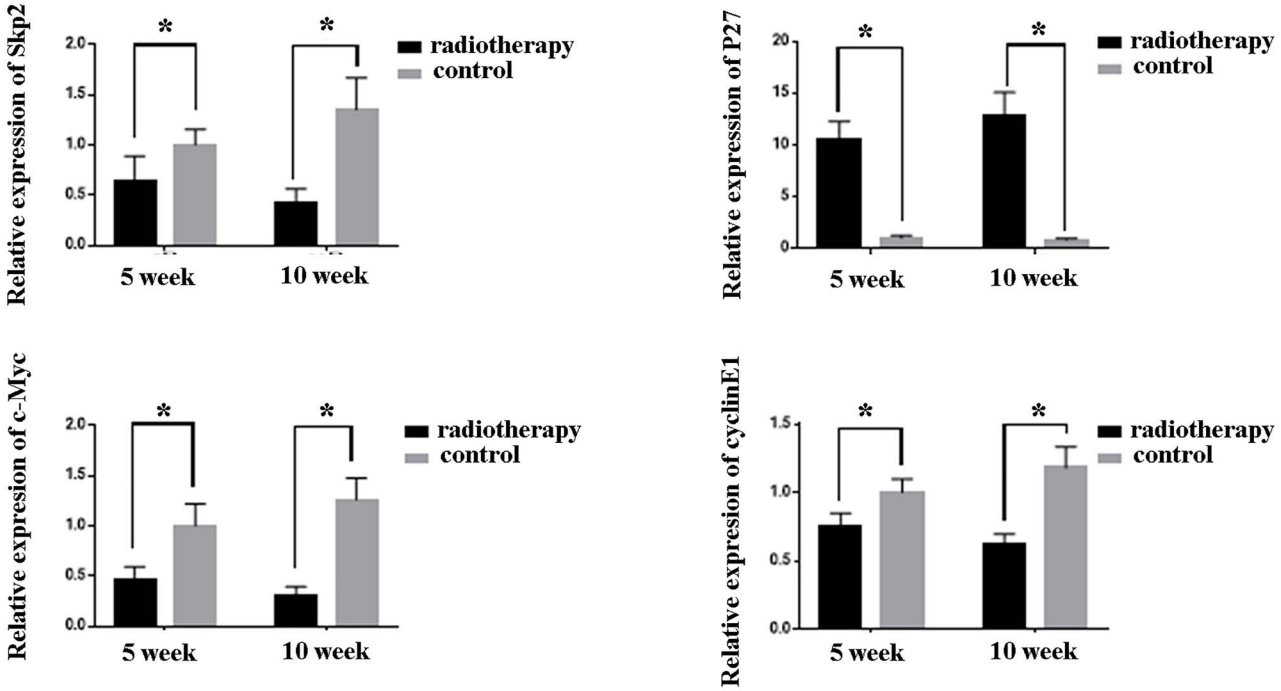


**Fig. 3. The influence of radiotherapy in mRNA level of proliferation-related factors. qRT-PCR was used to examine the expression of Skp2, P27, c-Myc and CyclinE1. * $P < 0.05$.**

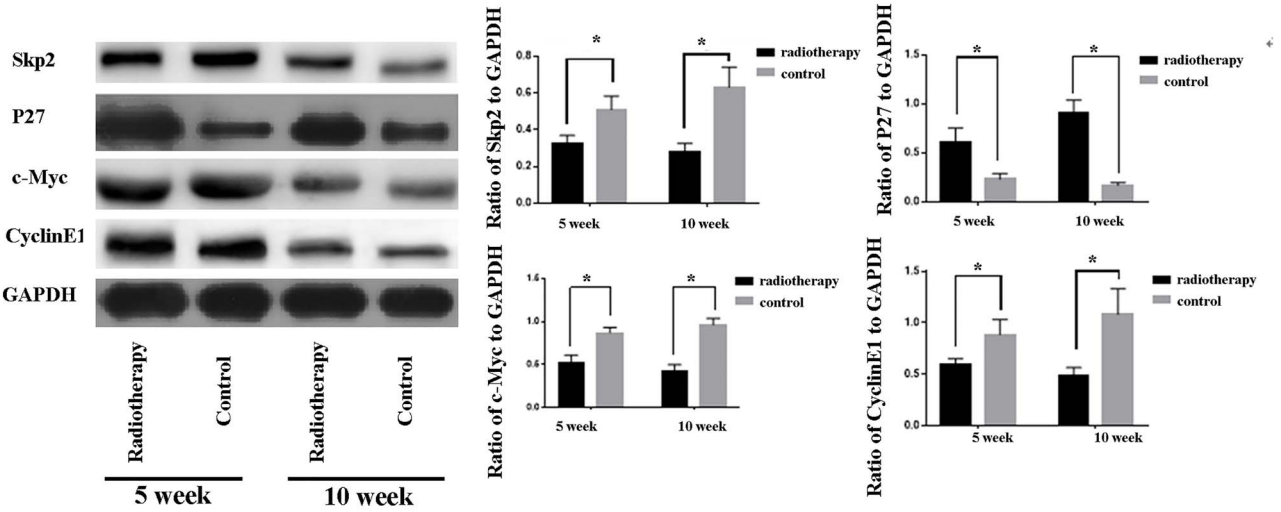


**Fig. 4. The influence of radiotherapy in protein level of proliferation-related factors. Western blotting was used to analyze the expression of Skp2, P27, c-Myc and CyclinE1. * $P < 0.05$.**

radiotherapy have not been evaluated this study. The long-term effect of radiotherapy on the vascular grafts was not investigated. Further study is still needed to investigate these questions.

In conclusion, postoperative external radiotherapy significantly reduces the intimal hyperplasia of artificial blood vessels by regulating c-Myc-Skp2-P27-CyclinE1 network. The above data provides a new idea and theoretical basis for the mechanism of radiation therapy on the intimal hyperplasia of artificial blood vessels. We will continue to study in depth and explain the mechanism and solutions of neointimal angiogenesis after artificial blood vessel reconstruction.

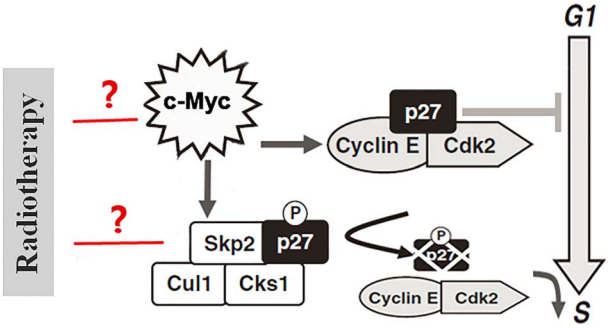


**Fig. 5. The schematic diagram of the possible mechanism of radiotherapy for neointimal hyperplasia.**

## AVAILABILITY OF DATA AND MATERIAL

The data sets used or analyzed during the current study are available from the corresponding author on reasonable request.

## CONFLICT OF INTEREST

The authors declare they have no conflicts of interest.

## FUNDING

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## ETHICAL APPROVAL

This study is approved by the Animal Care and Use Committee of Second Xiangya Hospital of Central South University.

## CONSENT FOR PUBLICATION

Informed consent was obtained from all individual participants included in the study.

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