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Original Research

Combined PCR and Q-RT-PCR technique for detecting chimerism in a non-human Primate vascularized osteomyocutaneous allografts model

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Abstract: Face transplantation and other composite tissue transplantation (CTA) are permissive to transplantation tolerance. The real reason, that composite tissue containing bone achieves transplantation immune tolerance more easily than the composite tissue without the bone is not clear. The chimerism may be the main mechanism in the progress of inducing the transplantation tolerance by CTA. We currently have established a non-human Primate Vascularized Osteomyocutaneous Allografts Model. To test the chimerism which comes from donor after the transplantation, we developed a method which combined reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qRT-PCR) technique using primers specific for Macaca fascicularis sex determination region on the Y chromosome (SRY) gene. With the method, we estimated the level of the chimerism.

Key words: Transplantation, RT-PCR, qRT-PCR, Chimerism.

Introduction

Currently organ allograft transplantation has emerged as an effective clinic procedure for end-stage organ failure patients. However, several immunosuppression-related problems remain. To successfully induce allograft tolerance and thus avoiding lifelong immunosuppressive therapy and deleterious side-effects is also a major challenge worldwide. Current successful approaches to inducing long-term stable mixed chimerism and allograft tolerance by using bone marrow cell transplantation require host conditioning with lethal irradiation and cytotoxic drugs (1-4). The concerns about the safety of cytotoxic drugs and/or irradiation have impeded the widespread clinic application of these strategies for organ transplantation (5-9). We have established the Vascularized Osteomyocutaneous Fibula Flap Transplantation Model which used non-human primate animals. The granted understanding of transplant tolerance using the unique vascularized bone marrow allograft model is likely to have an important impact on clinic organ transplantation (4, 10, 11). The chimerism was detected in mouse hind leg transplantation animal models in which the immune tolerance was inducted successfully. The chimerism might be the main mechanism in the progress of inducing the transplantation tolerance by composite tissue transplantation (CTA).

SRY gene is a gene for maleness found on the Y chromosome (12). It plays a key role in development of the testes and determination of sex (13) . SRY gene has little in common with sex determination genes of other model organisms (14) . The SRY gene provides instructions for transcription factor called the sex-determining region Y protein (15) which can differentiate the male samples from the others. If we can detect the SRY gene from the female receptor's DNA, we can say that there is chimerism in the receptor's circulation.

To achieve this goal, a sensitive and specific method to detect chimerism showing transplant tolerance is necessary. Although the Q-RT-PCR method has been used lot in transplant since early 2000s, methods which combined RT-PCR and qRT-PCR technique are rarely reported. In this study, we developed a method which combined RT-PCR and qRT-PCR technique for detecting chimerism in a non-human Primate Vascularized Osteomyocutaneous Allografts Model for Macaca fascicularis. The technique was applied to assess chimerism levels in rats transplanted with bone marrow.

Materials and Methods

Primate Animals

The Cynomolgus monkeys were obtained from Guangdong Landau Biotechnology Co., Ltd and maintained at the animal facilities in Tongji University. They received humane care in compliance with the guide for Regulations on the management of laboratory animal by the National Science and Technology Commission, China. The donor animals are male. The receptor animals were female.

Vascularized Osteomyocutaneous transplantation

We demonstrated the optimal vascularized osteomyocutaneous flap (cut range is up to) including the distal femoral 1/3, parts of muscle and subcutaneous tissue (flat down to the femur), the skin size was about

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the of 8*8cm. Vascularized osteomyocutaneous' blood supply came from the femoral artery branch and femoral vein reflux. In order to prevent muscle tissue of graft bleeding, swelling postoperative, we used electrosurgical knife and suture methods to hemostatic. The femoral artery and vein of vascularized osteomyocutaneous flap were end-and-end anastomosis to the femoral artery and vein of receptor. 9-0 sutures were used to finish the vascular anastomosis.

Collection of blood and extraction of genomic DNA

DNA was extracted from 0.5ml blood using TIANamp Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China). DNA was quantified spectrophotometrically with NanoDrop Spectrophotomer ND-100 (NanoDrop Technologies-USA), and then stored at -20°C to minimize acid hydrolysis. DNA extraction, RT-PCR and qRT-PCR reactions were conducted in different rooms.

Reverse transcription-polymerase chain reaction (RT-PCR)

PCR primers were designed specifically for M.fascicularis SRY gene (Genbank accession number Z26906.1) and GAPDH gene. The primer sequences and the expected sizes of PCR products were as follows: SRY, 5'- GTG GTC TCG CGA TCA GAGG-3'(forward) and 5'- TGG CCT GTA GTT TCT GTGCC -3'(reverse) (146bp); GAPDH, 5'-GAGTCAACGGATTTGGTCGTATTG-3'(forward) and 5'-CTCCTGGAAGATGGTGATGGGAT-3'(reverse)(200bp). The reaction system contained Dream Taq Green PCR Master Mix (2x) (Thermo, USA), H_2O(Nucleic acid free, lifetech, USA), 20pmol(2ul) of primers and 150(3ul) ng of DNA template which was extracted from receptor's blood. The total volume of reaction system was 20ul. GeneAmp PCR system 9700(Applied Biosystems, US) programmed as 1 cycle at 96°C for 2min, 30s at 94°C, 62°C for 45s and followed by 40s at 72°C for 40 cycles,7 min at 72°C for 1 cycle, and soak at 4°C.

Quantitative real-time PCR(qRT-PCR)

SRY was amplified from DNA of peripheral blood lymphocyte. DNA extracted from blood of chimeric monkey at 4 weeks after bone marrow transplantation and used as the DNA template for qRT-PCR using the set of primers. PCR was carried out on an Applied Biosystems 7500 Real-Time PCR Systems (Applied Biosystems, US) in a 20μL reaction mixture containing 10μL of 2x SYBR Green qPCR mastermixes (Power SYBR Green PCR Mastermix #43670659-Applied Biosystems), 1.5pmmol/ul of each primer, 7ul qPCR grade H_2O (AM9935, Ambion, USA) and 1ul DNA (10ng/ul). SRY, 5'- GCG AAA CTC AGA GAT CAG CA -3'(forward) and 5'- GGC CTG TAG TTT CTG TGC CT -3'(reverse)(92bp).

Construction of standard curve

Concentrations of DNA containing SRY gene were adjusted at 100 ng/μL using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). A tenfold dilution series of each DNA sample were used to construct the corresponding standard curves of SRY genes. Standard dilutions of each of the gene were assayed in triplicate. The Ct values were plotted against the logarithm of their initial template copy concentrations. Each standard curve was generated by a linear regression of the plotted points. From the slope of each curve, PCR amplification efficiency (E) was calculated according to the following equation:

$$E = 10^{-1/slope} - 1$$

Mixed genomic DNA gradient from donor and receptor blood of monkey was constructed according to the certain proportion. The DNA proportion of donor blood is 90%, 70%, 50%, 40%, 30%, 20%, 10%, 5%, 1% individually. Correspondingly, the DNA proportion from receptor was 10%, 30%, 50%, 60%, 70%, 80%, 90%, 95%, 99%. Then we generated the chimerism DNA standard curve. Then we can determine the proportion of chimerism in peripheral blood according the standard curve.

Quantitative PCR

To determine the absolute copy number of SRY genes in genomic DNA samples of monkey under study, the gene-specific primers were designed. Concentrations of all the DNA samples of test bulls were adjusted to 10ng/μl. The 'crossing point' or Ct values were determined by the software. All realtime PCR runs were performed in triplicate. Reaction for SRY gene comprised of 7ul qPCR grade H_2O (AM9935, Ambion, USA), 1.5pmmol/ul of each primer, 10μL of 2x SYBR Green qPCR mastermixes (Power SYBR Green PCR Mastermix #43670659-Applied Biosystems), and 1ul template DNA.

The following cycling conditions were employed for all the genes: pre-incubation at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C, 20 s at 60°C, and 1 s at 72°C. The fluorescence signal was measured at the end of each extension step at 72°C. After the amplification, a melting peak analysis with a temperature gradient of 0.1°C/s from 65 to 95°C was performed. Finally, the samples were cooled down to 40°C for 10 s.

Analysis of the PCR products

In order to test the authenticity of PCR products, the reaction products were electrophoresis. 10ul of the reaction product was resolved in 2% agarose gel and then stained with SYBR Green I for 1 h. Gel electrophoresis time was 17 minutes and voltage was 180V. The stained gels were directly scanned using a Bio imaging system (Bio rad). The intensity of the bands was quantified using ImageQuant software (Molecular Dynamics), which reflect the number of chimera in the blood.

Results

The primers designed for the PCR and qRT-PCR reaction were systemically tested for specificity, detection limit of target DNA in the presence of excess nonspecific DNA. Different primers were designed for the PCR and qRT-PCR reaction.

Specificity of the primers for SRY gene in RT-PCR

The SRY gene is only present on the Y chromosome. If there was the target band from receipt's DNA after transplantation, it meaned the chimerism in peripheral

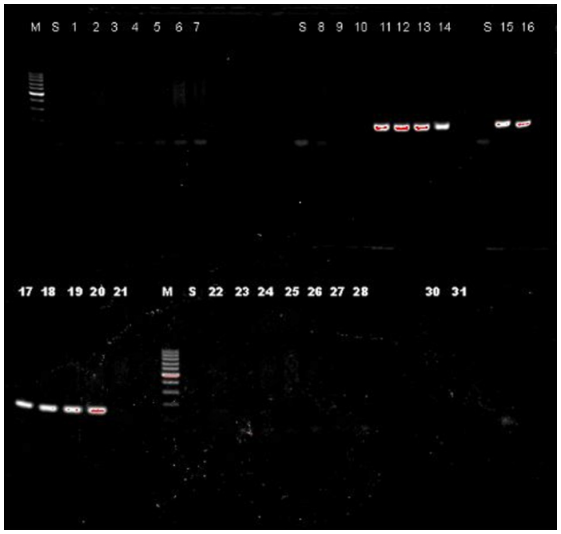

Figure 1. RT-PCR for SRY quantification.

blood. We get 30 cynomolgus monkey blood samples and extracted the DNA from the blood. The gender of the 30 cynomolgus monkey is unknown. After the PCR reaction, according to the PCR result, 11-20 are male, others are female in Figure 1. Our experimental results coincide with the actual situation. M stand for marker, S stand for H_2O as negative control. Line1 to 30 stand for 30 animals whose gender was unknown. Line31 is a female animal which has been convinced.

Specificity and sensitivity of the primers for SRY gene in qRT-PCR

As SYBR Green binds with double stranded DNA in sequence-independent manner, melting curves of reaction products were analyzed to confirm that only the specific products were amplified and circumvent any issues of non-specific fluorescence. Melting curve showed the single peak to indentify the specificity amplification of SRY gene in Figure 2. Five-fold dilution series of DNA ranging from 10ng to 0.1 ng (10^6 to 10copies/µl) for SRY were prepared for generation of standard curve for the sensitivity analysis of the SRY primer sets. The log concentration of DNA copies was plotted against the measured crossing point (Ct) values. Linear correlations between the logarithmic number of DNA copies and Ct values have been presented for SRY genes in Figure 3.

Construction of chimerism standard curve

The primer sets were then tested for sensitivity by performing qRT-PCR using a mixture of DNA containing decreasing amount (10 – 0 ng) of DNA from the donor monkey and increasing amount of DNA from the receptor monkey. Five-fold dilution series of DNA mixture ranging from 10ng to 0.01 ng (10^6 to 10copies/µl) for SRY were prepared for generation of standard curve for quantitative analysis. The log concentration of DNA copies was plotted against the measured crossing point (Ct) values in Table 1. Linear correlations between the logarithmic number of DNA copies and Ct values have been presented for SRY genes in Figure 4.

Quantification of SRY gene in test samples

DNA extracted from blood of chimeric monkey at 4 weeks after bone marrow transplantation and used as the DNA template for qRT-PCR using the set of primers. Relative copy concentrations in the donor and receipt monkeys for SRY genes were determined from the corresponding standard curves. The values of relative copy concentrations of Y- chromosomal genes in receptor groups of monkey have been compared in Figure 5. Results is shown in Figure 5, demonstrating that a significant number of was present in the recipient. The mean values of SRY in receptor monkeys are significantly ($P < 0.05$) higher compared to donor monkeys *of the donor group.*

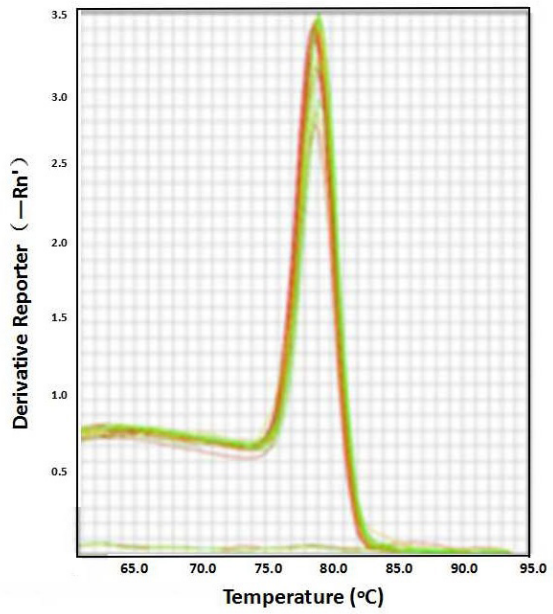

Figure 2. Melt curve of specific primers for SRY gene in qRT-PCR.

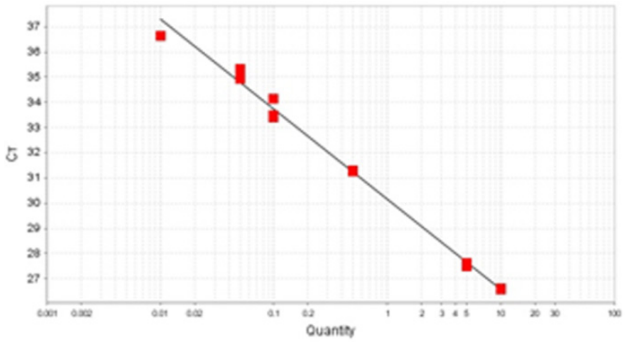

Figure 3. Construction of standard curve for SRY quantification.

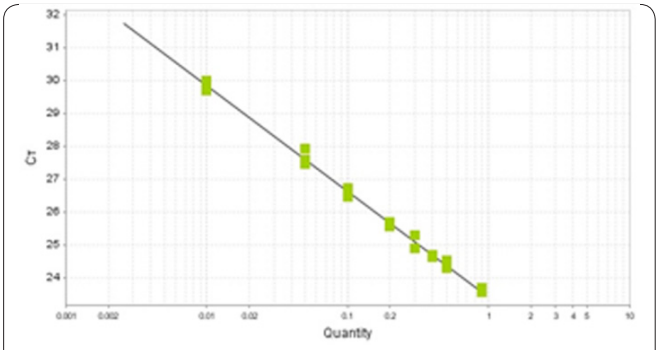

Figure 4. Standard curve of SRY quantification for chimerism.

Discussion

The organ allograft transplantation is the best way

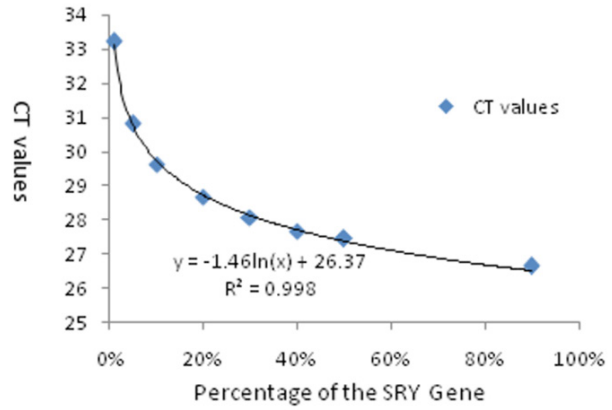

Figure 5. Relative copy numbers of SRY gene in genomic DNA of receptor monkey.

Table 1. Relative copy numbers of SRY gene in genomic DNA of receptor monkey.

Male DNA's proportion in total	CT value
90%	26.64±0.24
50%	27.44±0.21
40%	27.69±0.21
30%	28.09±0.19
20%	28.68±0.14
10%	29.62±0.25
5%	30.81±0.71
1%	33.21±0.70
F-DNA	36.6052475
H2O	Undetermined

for end-stage organ failure patients and has a long history. The patient must take Immune inhibitors to keep the organ alive. Immunosuppressive agents have produced many side effects. Immune tolerance has been transplantation research target since organ allograft transplantation began. The composite tissue transplantation has been for many years in the clinical application. The composite tissue transplantation (CTA) is easier to achieve transplantation tolerance (16-21). CTA transplantation has been used to induce transplantation tolerance in many animal models. The real reason of the immune tolerance is not clear (22). We hypothesized that the chimerism is the most important mechanism in the progress of inducing the transplantation tolerance. In order to test this hypothesis(23,24)

, we need to set up a sensitive and specific method to detect the chimerism. Priming specificity and efficiency are the major concerns in this progress.

The ideal primer should amplify the specific gene sequence from a single copy in genomic DNA which from the Macaca fascicularis. It is known that SRY is a single copy gene in Macaca fascicularis. If it is wanted that the method is more sensitive, the primers must be specific to multicopy genes, genomic repeats, and mitochondrial genes (25, 26). At the other side, the copies of the multicopy genes, genomic repeats could vary in different individuals (25). So we choose the single copy gene.

The SRY gene only exists in male people. Therefore, if we didn't detect the SRY copy in the blood but detect this copy after transplantation, it is sure that there is chimerism in the receptor's peripheral blood. Before transplantation, the receptor's DNA was used in the PCR and Q-RT-PCR reaction. It is sure that there isn't SRY gene copy in the receptor's peripheral blood before transplantation. The specificity of this method is very good.

Another advantage of this method, is its rapidity and simplicity. There are many methods to detect the chimerism. Many methods are complex and take a lot of time to complete the array. In the method, agarose gel was used to detect the chimerism after reaction. In addition, in Q-RT-PCR reaction SYBR Green technology allowed quantification of the PCR product without the lengthy processes. This method can detect the chimerism qualitatively and quantitatively.

The method which was described above was used to detect the level of chimerism using the DNA which is extracted from the receptor's peripheral blood after transplantation. In our experiment, the DNA was only from blood. But in this method, the DNA can be extracted from other tissue. This method can be used to detect chimerism in other solid tissues. As long as it can extract DNA from the organization, we can detect chimerism by using this method. We believe that this method will play an important role in the progress of finding the mechanism of immune tolerance.

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