Quantitation of Lymphangiogenesis Using the iCycler iQ™ Real-Time PCR Detection System and Scorpions Detection System

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Introduction

Lymphangiogenesis refers to the formation of new lymphatic vessels. This process may occur in normal developing tissues or in tumors. In addition to spreading by direct invasion and via the bloodstream, many malignant tumors, breast cancer for example, spread via the lymphatics once new connections have been established. For this reason, the biology of tumor lymphangiogenesis has important therapeutic implications. Methods to quantitate lymphangiogenesis have not previously been described. Measuring the density of lymphatic vessels has not been reliable due to a lack of sensitive lymphatic markers and antibodies. Furthermore, it does not indicate the rate at which the production of these channels is occurring. A more accurate method would be to measure the level of mRNA for lymphatic markers in tissues, since this correlates with the rate of lymphatic synthesis.

Lymphangiogenesis has not been studied in detail, primarily due to the lack of specific markers for lymphatic endothelium. However, a novel and specific lymphatic endothelial marker has been described, called LYVE-1 (Banerji et al. 1999). This is a surface epithelial receptor located on the lymph vessel wall. The receptor appears to be highly specific for lymph vessels and is completely absent from blood vessels (Skobe et al. 2001). By quantitating LYVE-1 mRNA in tissues, estimation of the rate of lymphangiogenesis in those tissues is possible. We report a new approach to enable the quantitation of LYVE-1 mRNA in breast cancer specimens using the iCycler iQ real-time PCR detection system.

Methods

Kits and Reagents

Primary human umbilical vessel endothelial cells from Clonetics were cultured in endothelial cell basal medium (also from Clonetics). The RNAzol RNA extraction reagent, reverse transcription kits, and PCR master mix were purchased from ABgene. Platinum supermix-UDG for quantitative PCR was purchased from Life Technologies.

The conventional PCR primers were designed by the authors and synthesized by Life Technologies. The TOPO TA cloning kit from Invitrogen was used for cloning. The agarose gel extraction kit and plasmid extraction kit were purchased from QIAGEN Ltd. The ABI PRISM BigDye terminator v 3.0 cycle sequencing kit with AmpliTaq DNA polymerase, FS was purchased from Applied Biosystems. The TaqMan reagents for quantitative PCR of β-actin were purchased from Applied Biosystems.

RNA Extraction and cDNA Preparation

Thirty frozen archival breast cancer specimens were homogenized and the total RNA extracted using the standard RNAzol procedure. The concentration of RNA was measured with a spectrophotometer. Equal amounts of cDNA were subsequently synthesized in 20 µl reaction mixtures by reverse transcription. The cDNA from human umbilical vessel endothelial cells was also synthesized in a similar fashion.

PCR and Sequencing

A pair of primers specific for part of the LYVE-1 gene sequence was designed to yield a DNA product of 925 bp. Conventional PCR was performed using the cDNA of human umbilical vessel endothelial cells and normal breast tissue together with the PCR mix and LYVE-1F and LYVE-1R primers to generate a 925 bp PCR product. The PCR product was sequenced to verify the LYVE-1 origin of the product.

LYVE-1 Cloning and Plasmid Preparation

The LYVE-1 PCR product was cloned using the pCR2.1-TOPO vector and One Shot *E. coli*, and the plasmid extracted from the *E. coli* using the plasmid mini purification kit. PCR was performed on the plasmid using the LYVE-1 primers to confirm that the isolated plasmid contained the correct DNA sequence. The concentration of the plasmid was determined by UV spectrophotometry. Following this, the concentration of the plasmid (copies/µl) was calculated using the known molecular weight of the plasmid plus insert. Serial dilutions of the plasmid were then made.

Quantitation of LYVE-1 in Breast Cancer Samples

The Scorpions probe/primers were formed by linking a forward primer and fluorescent probe using a PCR stopper (Oswel Research Products Ltd) as described by Whitcombe et al. (1999) to generate a 103 bp product from both the LYVE-1 plasmid and cDNA. Using the iCycler iQ detection system, the plasmid standards and breast cancer cDNA were simultaneously assayed in duplicate 15 µl reactions as follows: uracil DNA glycosylase supermix (7.5 µl), forward primer/probe (0.3 µl), reverse primer (0.3 µl), plasmid or specimen cDNA (1 µl), water (5.9 µl). PCR conditions were 95°C for 4 min, followed by 50 cycles of 95°C for 15 sec; 54°C for 20 sec; 60°C for 40 sec. Using purified plasmids as internal standards, the level of LYVE-1 cDNA (copies/µl) derived from the breast cancer samples was calculated. The sizes of the PCR products were verified on agarose gels.

Results

Quantitative Real-Time PCR Using In-House Standards

The standard curve generated by the LYVE-1 standards revealed a linear correlation between the log copy number of the purified LYVE-1 plasmid and the threshold cycle number, with a correlation coefficient of 0.987 (Figures 1 and 2).

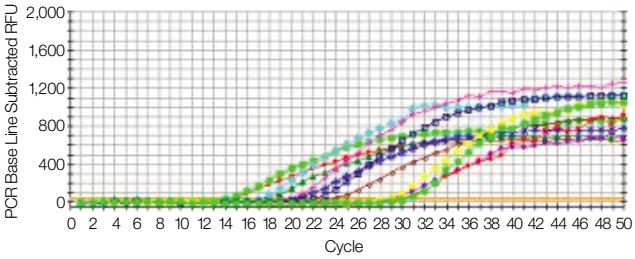

Fig. 1. Real-time PCR of six different concentrations of LYVE-1 plasmid standards (10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/µl), each performed 5 times.

Fig. 2. PCR threshold cycle number vs. log plasmid copy number.

Quantitation of LYVE-1 in Breast Cancer

LYVE-1 RNA was present in all 30 breast cancer specimens. The mean concentration of RNA in the specimens was 27.0 ± 12.6 copies/µl.

Discussion

Using a novel, specific lymphatic marker, LYVE-1, we were able to quantitate lymphangiogenesis using the iCycler iQ PCR detection system and Scorpions-based probes. Using these systems, a highly sensitive and specific detection and quantitation of LYVE-1 was possible. This is the first time that it has been possible to quantitate lymphangiogenesis indirectly.

The use of known concentrations of plasmid standards containing the desired sequence enables the concentration of the sequence in the original tissue to be quantitated, provided that the levels of RNA are initially standardized. It was found that LYVE-1 existed in all tested breast cancer specimens. Using this method, comparisons may be made between different subtypes of breast carcinoma to see whether the rate of lymphangiogenesis is related to cancer subtype. Other cancers may also be studied.

References

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Whitcombe D et al., Detection of PCR products using self-probing amplicons and fluorescence, Nat Biotechnol 17, 804–807 (1999)

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