THE DETECTION OF MDR1 GENE EXPRESSION USING FLUOROGENIC PROBE QUANTITATIVE RT-PCR METHOD

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ABSTRACT

Objective: To establish a fluoregenic probe quantitative RT-PCR (FQ-RT-PCR) method for detection of the expression of MDR1 gene in tumor cells and to investigate the expression of MDR1 gene in patients with lung cancer. Methods: The fluorogenic quantitative RT-PCR method for detection of the expression of MDR1 gene was established. K562/ADM and K562 cell lines or 45 tumor tissues from patients with lung cancer were examined on PE Applied Biosystems 7700 Sequence Detection machine. Results: the average levels of MDR1 gene expression in K562/ADM cells and K562 cells were (6.86±0.65)× 10^7copies/μg RNA and (8.49±0.67)×10^5 copies/μg RNA, respectively. The former was 80.8 times greater than the latter. Each sample was measured 10 times and the coefficient variation (CV) was 9.5% and 7.9%, respectively. Various levels of MDR1 gene expression were detected in 12 of 45 patients with lung cancer. Conclusion: Quantitative detection of MDR1 gene expression in tumor cells was achieved by using FQ-RT-PCR. FQ-RT-PCR is an accurate, and sensitive method and easy to perform. Using this method, low levels of MDR1 gene expression could be detected in 24% of the patients with lung cancer.

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Key words: Fluorogenic quantitative RT-PCR/MDR1, Expression/Real time, Detection

Competitive RT-PCR and quantative PCR using internal control as reference are usually used to detect the MDR1 gene expression in cancer cells. Both of these methods are end-point detecting method with considerable difference of product amounts between exponential and plateau phase. Therefore it is very difficult to exactly quantify primary template copies. Furthermore, there are some problems with competitive RT-PCR in practice, such as the PCR product contamination that result in false positive result, nonspecific amplification, low reproducibility and variations in the final results and the harmfulness of DNA staining dye EB. All of these affect its wide application in clinical studies. The newly established fluoregenic probe quantitative PCR(FQ-PCR) method is a great progress in the PCR technology. In this method, the PCR products are obtained in a closed tube and detected by the fluoregenic signal change. Therefore the PCR product contamination was greatly reduced. In addition, the exact number of the template copies can be determined by this method. This new method was first introduced into China by us in 1997 and was used to study the expression level of MDR1 gene in tumor cells. This report summarized the results of our studies.

MATERIALS AND METHODS

Cell Culture

K562/ADM drug resistance cell line was a gift kindly provided by Dr. Sun-shiren (Fujian Province

Tumor Hospital). K562 drug sensitive cell line was obtained from the Tumor Hospital affiliated to Sun Yat-sen University of Medical Sciences. These two tumor cell lines were cultured with the RPMI-1640 (Gibco) medium containing 20% new born cattle serum, 100 U/ml of penicillin and streptomycin. The cell cultures were incubated at 37° C with saturated humidity and 5% CO_2. For culture of K562/ADM drug resistant cell line, ADM was also added in the media at a final concentration of 3.8 μg/ml.

The Clinical Tissue Samples of Lung Cancer

Forty five postoperative lung cancer tissue samples from patients without chemotherapy were obtained from the Tumor Hospital affiliated to Sun Yat-sen University of Medical Sciences from May to September, 1998. These tissue samples were pathologically confirmed 19 cases of squamous cell carcinoma, 15 cases of adenocarcinoma, 4 cases of adenosquamous carcinoma, 4 cases of bronchioloalveolar carcinoma and 3 cases of lung metastases. Patients media age is 54.3 years old, between 39 to 71, 28 male and 17 female.

Extraction of Total RNA from Cells and Tissues

The total RNAs of the clinical samples, K562/ADM drug resistant cell line and K562 drug sensitive cell line were extracted as follows. 0.1-0.2g of fresh postoperative tissue of lung cancer or 10^6-10^7 cells were treated with 1 ml Trizole reagent. The total RNA was extracted according to the manufacturer's instruction. The RNA was quantified with UV spectrophotometer at OD260 and 280nm (the ratio of OD260/280 must be =2.0).

Design, Synthesis, and Purification of Primers and Probes

The primers for amplification of MDR1 gene were designed according to reference 1.The forward primer was: 5'-CCCATCATTGCAATAGC- AGG-3'. The reverse primer was: 5'-GTTCAAA-CTTCTGCTCCTGA-3'. The length of amplified DNA was 167 bps. The oligonucleotide probe was 5'-CGC-TACTGAAGCAATAGAAAACTT-3'. These primers and probes were synthesized and purified in our laboratory. The probe was labeled with 6-carboxy- fluorescein (FAM) at the 5'end and 6-carboxy-tetya-methylrhodamine, (TAMRA, PE.com) at the 3'end.

Reverse Transcription of RNA to cDNA

Two μg total RNA from the cells of K562/ADM drug resistance and K562 drug sensitive cell lines or fresh tissues of lung cancer was reverse-transcribed in 10μl reaction mixture including 50mmol/L Tris-HCl (pH8.3), 40mmol/L KCl, 7mmol/L MgCl2, 1mmol/L DTT, 0.01% BSA, 1mmol/L dNTPs, 20U MMLV reverse transcriptase (Shang hai Sangon Inc.), 10URNase inhibitor (Huamei Biotechnical Inc.), 3μmol/L specific reverse primer at 37℃ for 60 min followed by 95℃for 5 min. The synthesized cDNAs were stored at -20℃.

Quantification of Positive Standard cDNA Clone

The PCR amplified 167bps mdr1 gene cDNA was cloned into pUC18. The copy number of recombined plasmid was calculated by measuring its OD260/280 and adjusted to 10^{10}/μl.

The Fluoregenic Probe Quantitative RT-PCR (FQ-RT-PCR)

The principle of fluoregenic probe quantitative PCR is based on regular PCR and specific hybridization of a probe to the amplified DNA sequence. The two ends (5' and 3') of the probe were labeled with different fluorescent dyes. 5' end fluorescent dye is a high energy one called reporter and 3' end fluorescence dye is low energy one called quencher. There is no fluorescent signal can't be detected when the fluoregenic probe is kept intact, because 5' end reporter energy is absorbed by the 3' end quencher. But during the extension phase the probe is cleaved by the nuclease activity of the Taq polymerase. This cleavage generates fluorescent reporter signal, which is proportional to PCR product accumulation. The exact DNA copy number of amplified sequence can be obtained by fluorescent signal intensity detected by a machine. The cycle threshold (CT) is used as standard detecting point, which is located at the start point of the exponential phase. The FQ-PCR reaction system includes 10 mmol/L Tris-HCl (pH8.3), 2 mmol/L MgCl2, 50 mmol/L KCl, 0.2 mg/ml gelatin, a pair of mdr1 primers each at 3 μ mol/L, the fluoregenical probe at 2 μ mol/L, Taq DNA polymerase at 3U, and cDNA template in total of 50 μl volume. At the same time the quantitative standard cDNA plasmid was serially diluted in a system with ABI PRISM 7700 modal machine to obtain a standard curve for quantitating the samples. The reaction condition is: pre-denaturalization at 93℃ for 3 min, then 40 cycles of 93℃ for 40 sec, 55℃ for 1 20 sec.

RESULTS

Preparation of Standard Template