

RESEARCH ARTICLE

Multiplex Real-time PCR for RRM1, XRCC1, TUBB3 and TS mRNA for Prediction of Response of Non-small Cell Lung Cancer to Chemoradiotherapy

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Abstract

Background: This study was aimed to establish a novel method to simultaneously detect expression of four genes, ribonucleotide reductase subunit M1 (RRM1), X-ray repair cross-complementing gene 1 (XRCC1), thymidylate synthase (TS) and class III β -tubulin (TUBB3), and to assess their application in the clinic for prediction of response of non-small cell lung cancer (NSCLC) to chemoradiotherapy. **Materials and Methods:** We have designed four gene molecular beacon (MB) probes for multiplex quantitative real-time polymerase chain reactions to examine RRM1, XRCC1, TUBB3 and TS mRNA expression in paraffin-embedded specimens from 50 patients with advanced or metastatic carcinomas. Twenty one NSCLC patients receiving cisplatin-based first-line treatment were analyzed. **Results:** These molecular beacon probes could specially bind to their target genes in homogeneous solutions. Patients with low RRM1 and XRCC1 mRNA levels were found to have apparently higher response rates to chemoradiotherapy compared with those with high levels of RRM1 and XRCC1 expression ($p < 0.05$). The TS gene expression level was not significantly associated with chemotherapy response ($p > 0.05$). **Conclusions:** A method of simultaneously detecting four molecular markers was successfully established and applied for evaluation of chemoradiotherapy response. It may be a useful tool in personalized cancer therapy.

Keywords: Multiplex quantitative real-time polymerase chain reaction - chemotherapy related markers - cancers

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide. Eighty-five percent of lung cancer patients are diagnosed yearly with non-small cell lung cancer (NSCLC) (Sun et al., 2014). Although chemotherapy has been playing an important role in improving survivals of lung cancer patients as a part of multimodal therapy, the widely spread drug resistances and the seriously unexpected side effects, which always resulted in low survival rate and/or low response rate, launched an emergent requirement for novel therapies of cancers. Alternatively, we could optimize personalized cancer therapies in order to enhance patient's survival rate. A lot of cancer markers have been identified for tailored treatment of cancers, including platinum targeting markers such as excision repair cross-complementing 1 (XRCC1), X-ray repair cross-complementing gene 1 (XRCC1), ribonucleotide reductase subunit M1 (RRM1) and class III β -tubulin (TUBB3), and 5-FU targeting markers such as thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD) and breast cancer susceptibility gene 1 (BRCA1).

Molecular beacons (MBs) are labeled single-stranded oligonucleotide which possess a stem-and-loop structure (Tyagi et al., 1996; Sokol et al., 1998). The loop is single strand complemented to its target sequence, while the stem is double strand formed by two anti-complementary arms of 5 to 7 bp to stabilize the loop. The end of one arm was labeled with fluorophore, and the end of the other arm was attached with a quencher. The binding of the loop to its target sequence opened the stem and removed the quencher from the fluorophore resulting fluorescence (Stryer, 1978). MB generating signals only in the presence of the target. Stem-loop hairpin probes have showed better specificity in gene detection compared to linear oligonucleotide probes (Roberts et al., 1991), and thus present a higher sensitivity and signal-to-background ratio than Taq-Man probes. Shared-stem molecular beacons were also found to yield better performances than Taq-Man probes (Wang et al., 2005). The molecular beacon therefore enable the detection of multiple targets in the same tube.

Changes in gene expression pattern occurred during cancer development offered clues for cancer diagnosis,

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therapy, and prevention (Leng et al., 2012). Former studies focusing on single gene failed in these purpose, but the others taken multiple genes were always expensive and time-consuming. So more economical methods which could simultaneously detect multiple gene expressions were required. In this study, a multiplex RT-PCR assay was designed to examine the expression of XRCC1, RRM1, TS and TUBB3 in tumor samples from patients using molecular beacon.

Materials and Methods

Patients and samples

Fifty patients included in this study were registered with locally advanced cancers in affiliated ZhongDa hospital of Southeast University between 2010 and 2012. There were 23 males and 27 females, aged 45-84 years old, and among them there were eight cases (16%) of colorectal cancers, ten cases (20%) of gastric cancers, eleven cases (22%) of esophageal and twenty one cases (42%) of lung cancers. In brief, sample patients for this study met all the eligibility criteria as follows, (a) diagnosed with cancer by biopsy; (b) no previous chemotherapy; (c) qualified performance status, hepatic, hematological, and renal function for chemotherapy. Informed consents from each patient were collected and this study had been approved by the ethics committee of our hospital.

Treatment

Patients were primarily evaluated by physical examination, performance status, computed tomographic scans of the chest and abdomen, complete blood count, and pelvic magnetic resonance imaging. Twenty one of these patients received at least two cycles of adjuvant chemotherapy within three months after surgery, and all patients received GP (Cisplatin 40 mg/m² from day 1 to day 4, Gemcitabine 1800 mg/m² on days 1,8) every 3 weeks. All tumor tissue specimens were fixed in 10% formaldehyde solution and embedded in paraffin (FFPE) until analysis was available.

RNA extraction and cDNA synthesis

FFPE samples from 50 patients were collected, and slice sections of 7 μm thickness were prepared. The sections with paraffin were dewaxed in 100% xylene for 10 minutes, and hydrated with 100%, 95%, 90%, 80%, and finally 75% ethanol solutions, sequentially. Total RNA were extracted using Trizol Kit (Invitrogen, California, USA) following the manufacturer's instructions.

Total RNA were quantified, and visualized by 1.5% agarose gel electrophoresis. An amount of 50 ng total RNA were reverse transcribed for single-strand cDNA using oligo (dT)15 primer and Superscript II (Takara, Tokyo, Japan) and scaled up to a final volume of 10 μL. Reverse transcription reaction was performed at 85°C for 15 minutes, followed at 37°C for 5 seconds.

Design of probes and primers

We designed and validated four sets of primers and MB probes on the most conserved regions with Primer 5 and

Beacon 7 software, synthesized by Invitrogen company (Invitrogen, California, USA). All the alignments were carried out using Mfold (Version3.0). The different target genes were quadruply multiplexed by PCR. Primer for each gene are as following: 1) TS sense 5'-AGTGGAGGCATTTTGGGGCAGA-3', antisense, 5'-TGGCAGGACAGCTCACTGTTCA-3'; 2) RRM1 sense, 5'-AAACGCCATCGCCCCATTGGAA-3', antisense, 5'-ACAGCTGGCTTCCAGAGCACCATA-3'; 3) XRCC1 sense, 5'-ACACTTACCGAAAATGGCGGGC A-3', antisense, 5'-TTGCTGTAGGGCTGGCTGCAA -3'; 4) TUBB3 sense, 5'-GCCTGACAATTCATCTTT-3', 5'-TCACACTCCTTCCG CACCA-3'; 5) β-actin sense, 5'-CACTCTTCCAGCCTTCCTTCC-3', antisense, 5'-TGCCAGGGTACATGGTGGT -3'.

The specific probe of 5'-CGCGAAGATTATTCAGGA CAGGGAGTCGCG-3' labeled with the fluorescent reporter dye Cy5 at the 5' end was for TS, 5'-CGCGATCC TGATGAGATACCCTTTCGCG-3' with FAM was for RRM1, 5'-CGCGATGGAGAAGGAGGAGCAGATTCG CG-3' with HEX was for XRCC1, and 5'-CGCGAAGG GTCACTACAC GGAGGGGTTCGCG-3' labeled with TAMRA was for TUBB3. All the probes were labeled with the quencher dye DABCYL at the 3' end. The stem sequences of probes were underlined.

Multiplex PCR strategy

IQ 5 Multicolor Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) was used in this study.

The PCR cycling was initiated with 5 min of denaturation at 95°C, followed by 40 cycles of denaturation for 45 sec at 95°C, annealing for 30 sec at 50~60°C and amplification for 30 sec at 72°C. The PCR was finished with an extra elongation at 72°C for 5 min. PCR solution formula included 0.5 units of DNA Taq polymerase (Takara, Tokyo, Japan), 1 μL each primer, 1 μL cDNA, 2 μL dNTPs, 2.5 μL 10×PCR buffer, 4 μL MgCl₂, 1 μL each MB probe and added ddH₂O in a total volume of 25 μL. Verify PCR products by 1.5% agarose gels in the same reaction tube. Then we selected one of the best annealing temperature is 50°C. In the same cycle condition, β-actin reactions were carried out in a total of 25 μL volumes containing 10 pmol of each primer, 12.5 μL SYBR Green Mix, 1 μL template DNA and 9.5 μL ddH₂O of another tuber.

All samples were tested by a previously described SYBR Green-based real-time PCR method using RRM1, XRCC1, TS and TUBB3 primers, respectively. After the completion of 40 PCR cycles, the four genes unique melting-curve data were obtained by continuous fluorescence acquisition from 65 to 95°C, with a thermal transition rate of 0.1°C/s.

Development of plasmid constructs

PCR fragments were purified using Wizard DNA purification Kit (Promega, Madison, USA), then cloned into pMD18 vector using TOPO TA cloning kit (Invitrogen, USA). After overnight incubation at 4°C, the ligation mixture was transformed into competent Escherichia coli (DH5α) and grown overnight on agar plates containing ampicillin and X-gal. Positive clones

were identified using blue-white screening. Five milliliter LB-broth cultures containing single colonies were grown up overnight, shaken at 200 rpm at 37°C and subsequently purified using the QIA prep Spin Miniprep Kit (Qiagen, Dusseldorf, Germany). Plasmids DNA were dissolved in 50µl TE buffer for sequencing in Sangon company (Shenggong, shanghai, China).

Relative mRNA levels of target genes

Relative mRNA levels were determined by the standard curve method. The standard curves for TS, RRM1, XRCC1, TUBB3 and β-actin mRNA were generated using fivefold serially diluted solutions of plasmid clones inserted by either TS, RRM1, XRCC1, TUBB3 or β-actin cDNA as templates. The argument Ct (threshold cycle) was defined as the fractional cycle number at which the fluorescence exceeded the threshold level, that is, a higher Ct value meant that more PCR cycles are required to reach a certain level of detection.

The amount of target genes expression was calculated from the standard curve, and the quantification of each cDNA in samples was performed using the β-actin expression level as an endogenous control. Finally, the mRNA levels for each target genes were expressed as a ratio to the β-actin mRNA. Real-time PCR assays were conducted in duplicate for each sample.

Multiplex real-time RT-PCR using MB

After removing the quencher, FAM, Cy5, HEX and TAMRA would emit fluorescence of different wavelength which could be observed by synchronous scanning fluorescence spectrometry, separately. Thus, the simultaneous detection of XRCC1, RRM1, TS and TUBB3 could be achieved by measuring fluorescence signals of FAM, Cy5, HEX and TAMRA separately.

The PCR was carried out as described above. The fluorescence intensity was obtained every cycle at 50°C. All samples were run in duplicates.

Methodological evaluation

The specificity of the new method was determined by detecting the melting temperature of 50 samples both the multiplex real-time PCR assay and its corresponding SYBR assays.

The sensitivity of our method was determined by running the PCR with serial template concentrations of plasmid DNA ranging from 4×10^{12} to 4×10^2 , from which, we defined the detection limit.

The intra- and inter-assay coefficients of variation were used to assess the accuracy of each experiment. The intra-assay coefficients of variation was determined from duplicate values within all runs of the same assay. The inter-assay coefficients of variation were calculated from the mean values of duplicate assays performed in different days.

Evaluation of chemotherapy response

Chemotherapeutic response was clinically evaluated by measuring the change in tumor size or recurrent region. The specific evaluations were as follow: (a) CR (complete response), disappearance of all known disease; (b)

PR (partial response), $\geq 50\%$ lessen in tumor size; (c) no change, $< 50\%$ decrease or $< 25\%$ increase in tumor size; and (d) progressive disease, $\geq 25\%$ increase in tumor size or appearance of new lesions. In this study, CR and PR were defined as responders, and no change and progressive disease were defined as non-responders.

Statistical analysis

The median relative mRNA levels standardized for β-actin were selected as cut-off value of high and low level genes expression. Associations between mRNA levels and clinicopathological characteristics were assessed by statistical significance using a the χ^2 test or Fisher's exact test (Software Package SPSS for Windows, Version 11.0; SPSS, Chicago, IL). OS was calculated from the date of surgery to the date of death due to chemoradiotherapy. Survival was evaluated using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups. *p* value < 0.05 was considered significant statistically.

Results

Identification of multiplex PCR products

PCR product amplified by four pairs of primers in a multiplex PCR system were visualized on 1.5% agarose gel. There were four bands of 331, 220, 147 and 117 bp corresponding to XRCC1, TS, RRM1 and TUBB3 respectively (Figure 1), which were consistent with their theoretical size. No bands were seen from the negative control.

Sensitivity and Specificity of Multiplex Real-Time PCR Assay

Figure 2 (A1) showed the PCR amplification plots of β-actin with different concentrations of template. Figure 2 (B1, C1, D1, and E1) showed the PCR amplification plots of each gene with different concentrations of template in a single tube. The linear fits for each gene were showed in Figure 2 (A2, B2, C2, D2 and E2).

The intra- and inter-assay variations are shown in (Table 1). For the XRCC1, RRM1, TS and TUBB3 assays Ct values had a mean intra-assay CV below 1% and a

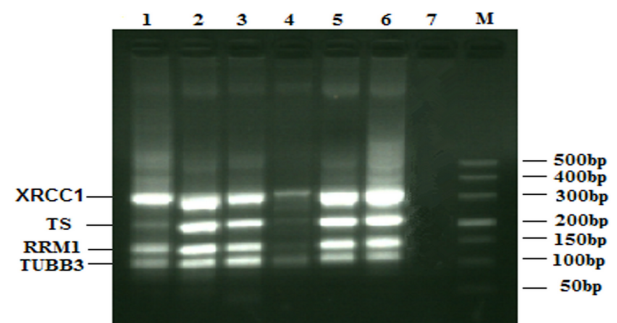


Figure 1. Multiplex PCR for the XRCC1, TS, RRM1 and TUBB3 genes. The PCR products were separated over an 1.5% agarose gel. Lines: M, molecular size marker; 7, negative control; 1 to 6 temperature gradient of four genes PCR products, temperature followed is 60°C, 58.2°C, 56.8°C, 54.5°C, 52.3°C and 50°C

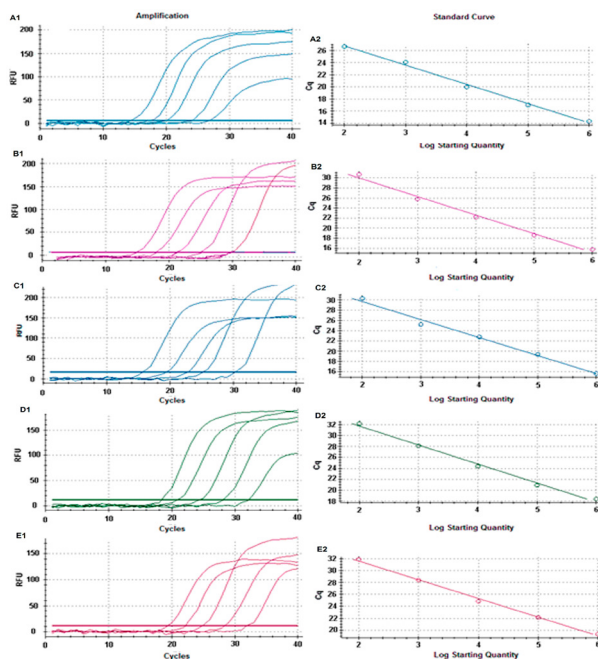


Figure 2. PCR Amplification of Genes. (A1-E1) The PCR Amplification plots of β -actin, TS, RRM1, XRCC1 or TUBB3 gene using different template concentrations of a 10-fold dilution series (102-106, from left to right). The released reporter fluorophore is plotted as a function of the amplification cycle number. (A2-E2) Standard curve plotting log starting copy number versus Ct: $Y=-3.183X+41.626$, with $R^2=0.998$; $Y=-3.427X+41.626$, with $R^2=0.997$; $Y=-2.176X+35.372$, with $R^2=0.991$; $Y=-3.055X+40.39$, with $R^2=0.995$ and $Y=-3.133X+37.837$, with $R^2=0.996$, respectively

mean inter-assay CV below 10%. Calculated plasmid copy number had mean intra-and inter-assay CVs below 30%.

Patient characteristics and survival

In this study, three genes mRNA expression levels were successfully measured for all patients. The median relative genes mRNA expression levels were as following, TS: 1.29 (range,0-1.8), RRM1: 1.16 (range0-1.62), XRCC1: 1.295 (range0-2.14). The values of samples were divided into two groups according to their mRNA levels. No significant differences of clinicopathological characteristics were found between the high and low mRNA level groups (Table 2). Medians of OS were 25.6 months (range: 8.3-59.4). As shown in Figure 3, when treated with Cisplatin and Gemcitabine, patients with low RRM1 and XRCC1 expression had increased OS compared to those with high expression in the chemoradiotherapy subgroup ($p=0.002$ and $p=0.009$, respectively). But TS expression was not associated with OS ($p=0.451$).

Table 1. Overall Mean Intra-and Inter-Assay CV Percentage Based on Ct values and Calculated Plasmid Copy Numbers (CPCN) of Standards

Assay	Mean intra-assay CV		Mean inter-assay CV	
	Ct	CPCN	Ct	CPCN
RRM1	0.52	26.2	3.8	19.4
TS	0.68	20.8	5.2	22
β -tubulin III	0.63	23.5	2.6	25.3
XRCC1	0.7	27.3	4.7	18.7

Table 2. Clinicopathological Characteristics Associated with Genes mRNA Expression

Variable	Number	RRM1		P	XRCC1		P	TS		P
		High level	Low level		High level	Low level		High level	Low level	
Gender										
Male	23	14	9		12	11		7	16	
Female	27	8	15	0.204	17	10	0.663	19	8	0.152
Age(median 58)										
<58	20	8	12		11	9		7	13	
>58	30	13	17	0.07	14	16	0.272	8	22	0.503
Histologic grade										
Well and moderately	22	11	11		9	13		15	7	
Poorly differentiated	28	18	10	0.165	13	15	0.465	12	16	0.311
Lymphatic invasion										
Present	29	16	13		11	18		19	10	
Absent	21	7	14	0.095	15	6	0.101	11	10	0.083
Stage										
I~II	18	8	10		9	9		12	6	
III~IV	32	15	17	0.535	18	14	0.325	20	12	0.225

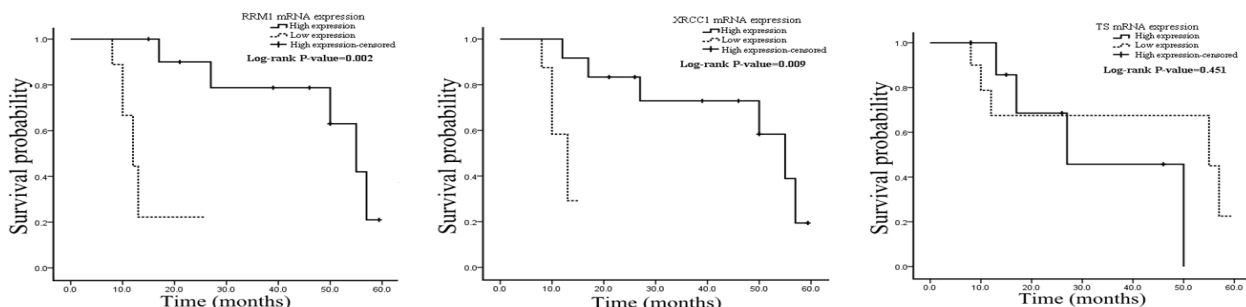


Figure 3. Overall Survival (OS) in 21 NSCLC Patients Receiving Cisplatin and Gemcitabine Treatments according to RRM1, XRCC1 and TS mRNA levels

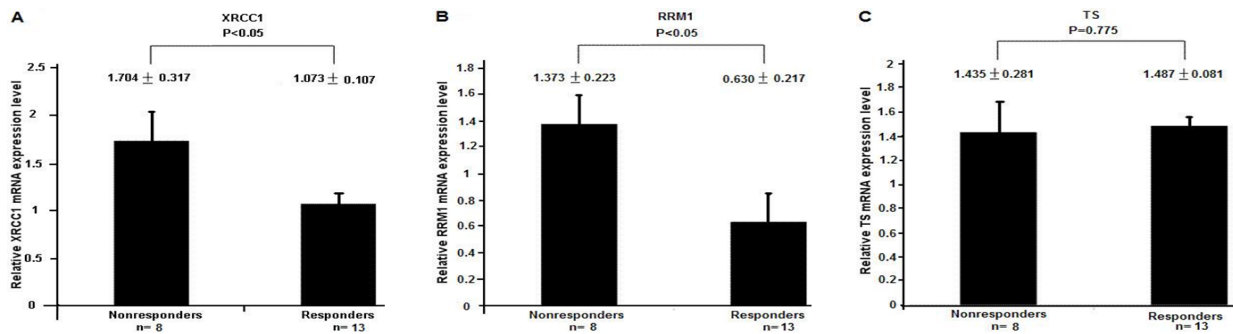


Figure 4. Comparison of Genes mRNA Expression Levels and Their Ratios between Responders and Nonresponders to Cisplatin and Gemcitabine Treatment. A) XRCC1; B) RRM1; C) TS

Genes mRNA expression and clinical response to Cisplatin and gemcitabine treatment

For myelosuppression and infectious complications, only twenty one (42%) patients received cisplatin-based concurrent chemotherapy. The treatment response was evaluated by CT imaging at the end of treatment.

Among these 21 patients, thirteen showed response (CR+PR) to cisplatin and gemcitabine with a response rate of 61.9%. RRM1 mRNA levels of responders to cisplatin and gemcitabine (0.846 ± 0.338) were significantly ($p=0.027$) lower than those of non-responders (1.372 ± 0.223), XRCC1 mRNA levels of responders to cisplatin and gemcitabine (0.948 ± 0.442) were also significantly ($p=0.037$) lower than those of non-responders (1.582 ± 0.293), but TS mRNA levels of responders to cisplatin and gemcitabine (1.366 ± 0.178) were less significant ($P=0.775$) than those of nonresponders (1.435 ± 0.281) (Figure 4).

Discussion

Along with the development of theoretical and practical chemotherapy for tumors, we have already known the combination of two drugs such as platinum and non-platinum is the standard first-line therapy for NSCLC patients (Crino et al., 2010). During the targeted therapy era, targeted drugs, such as gefitinib and erlotinib, can effectively reduce the incidence of brain metastases in patients with NSCLC (Zeng et al., 2012)

Several studies showed that RRM1, XRCC1, TUBB3 and TS expression in tumor cells are involved in platinum and non-platinum (Rabik et al., 2007; Takezawa et al., 2011; Xie et al., 2013). The goal of this study was to establish a new method to detect four biomarker genes expression and to choose effective drugs to guide personalized cancer therapies. In this study, we have investigated RRM1, TS, TUBB3 and XRCC1 mRNA expression in patients.

TS plays an important role in DNA biosynthesis by catalyzing the methylation of dUMP to dTMP (Grem et al., 1987). As important group of antineoplastic drugs, 5-fluoropyrimidines and pemetrexed could target on TS, and are widely used in the treatment of gastrointestinal tumors, breast tumors, and epithelial tumors of the upper aero digestive tract (Grem, 1988; Goyle et al., 2006). Previous studies performed on several cancers have demonstrated that the TS expression level predicted the response to 5-FU

or pemetrexed chemotherapy (Foekens et al., 2001). Higher TS mRNA levels in tumors have generally been associated with poor clinical outcome in patients receiving 5-FU or pemetrexed chemotherapy regimens (Popat et al., 2004; Perumal et al., 2006). In our study the cancer patients received a combination of cisplatin and gemcitabine, TS mRNA level showed a insignificantly association with chemotherapy response ($p>0.05$).

Microtubules are a complex consisting of tubulin dimmers and a variety of microtubule-associated protein (Seve et al., 2005). Studies in lung cancer have shown that high expression levels of the brain-specific TUBB3 are associated with paclitaxel resistance in a preclinical setting which has been further proved in patients with advanced NSCLC (Zhang et al., 2013). In this study, TUBB3 gene was detected successfully only in four samples, therefore we couldn't evaluate the efficacy of chemotherapy based on a few numbers. It might be caused by the limitation of different cycles of adjuvant chemotherapy taken by patients and other interferences like number of samples and only one clinical center involved in our study.

RRM1 is a component of ribonucleoside-diphosphate reductase located on chromosome segment 11p15.5 (Bepler et al., 2002), which is the predominant cellular determinant of the efficacy of gemcitabine (Bepler et al., 2006). Rosell et al. (2004) found RRM1 mRNA level is a crucial predictive marker for the survival of the advanced NSCLC patients receiving gemcitabine/cisplatin. Clinical studies in non-small cell lung patients who received gemcitabine-based therapy revealed that patients with low levels of RRM1 expression showed a better response and a longer survival time than those with high RRM1 levels (Zhang et al., 2012; Xie et al., 2013). Substantial evidence indicates an important role for XRCC1 in single-strand break repair (SSBR) and base excision repair pathway (BER) (Fan et al., 2004). The amount of XRCC1 transcription has shown a significant correlation with cisplatin resistance among NSCLC cell lines. There is limited data on XRCC1 expression in tumors, despite the fact that at least in NSCLC cell lines increased XRCC1 mRNA is significantly associated with cisplatin resistance (Weaver et al., 2005). Our study are similar to this result that only patients with low RRM1 and XRCC1 mRNA levels have complete response to chemotherapy treatment ($p<0.05$).

The most important advantage of our method was that we could simultaneously detect four genes by the

peculiar molecular beacons. We developed a multiplexed DNA detection method based on guanine-quenching composite MBs by synchronous fluorescence analysis. Compared with previously reported methods, the proposed method has some advantages including easy synthesis of composite MB probes, low cost, fast binding kinetics, good precision and a low detection limit, and can markedly distinguish the perfectly complementary sequence from the mismatched sequence.

Fifty patients were evaluated in this study, but according to the clinical chemotherapy qualification only twenty one patients received the combination treatment of cisplatin and gemcitabine. We found that NSCLC patients with low RRM1 and XRCC1 mRNA level had a significantly higher rate of complete response than patients with high levels ($p < 0.05$). According to the spearman rank correlation analysis, gene mRNA level represented an independent role in predicting complete response to chemotherapy.

In conclusion, this is first study to detect four genes in a single tube by MB probes. Our findings indicate that XRCC1 and RRM1 can predict chemotherapy response in NSCLC patients. Further investigation is required to determine whether these assays are sufficiently reliable to use routinely as a basis to select specific patient treatments.

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