Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Combined detection of p53, p16, Rb, and EGFR mutations in lung cancer by suspension microarray

Y. Ye[1], D. Wang[1], C. Su[1], T. Rong[2] and A. Guo[1]

[1]Cancer Center of People's Liberation Army, Nanjing, PR, China
[2]Department of Thoracic Surgery,
Sun Yat-sen University Cancer Center, Guangzhou, PR, China

Corresponding author: D. Wang
E-mail: wangdong_nj001@hotmail.com

Genet. Mol. Res. 8 (4): 1509-1518 (2009)
Received August 26, 2009
Accepted October 15, 2009
Published December 23, 2009

ABSTRACT. Mutations of some contributing factors (p53, p16, Rb, and EGFR) are believed to affect diagnosis and drug resistance of lung cancer. We evaluated the efficacy of a multimarker panel for molecular diagnosis of lung cancer, using a high-throughput suspension microarray. One hundred and twenty-five lung cancer specimens and 30 tumor-free lung tissue samples were assayed by multiplex polymerase chain reaction with specific probes designed to detect hot-spot mutations in p53, p16, Rb, and EGFR. The mutation rates of p53, p16, Rb, or EGFR in the lung cancer specimens were 36.8, 15.2, 11.2, and 18.4%, respectively. Inclusion of four markers elevated sensitivity to 68.0%. The specificity and accuracy of four-marker detection were 90.0 and 72.3%, and the mutation rates of this panel in stage I, stage II and stage III disease were 62.2, 65.9 and 75.0%, respectively. Mutation at p16 occurred more frequently in non-small cell lung cancer (19.3%) than in small cell lung cancer (5.4%); while the mutation rate of Rb was 32.4% in small cell lung cancer versus 2.3% in non-small cell lung cancer. We conclude that simultaneous detection of p53, Rb, p16, and EGFR in a

suspension microarray facilitates rapid diagnosis of lung cancer.

Key words: Lung cancer; Diagnosis; p53; p16; Rb; EGFR

INTRODUCTION

Lung cancer is one of the leading causes of cancer death throughout the world. Over the last two decades, molecular studies have focused on the identification of target oncogenes and tumor suppressor genes, which are activated and inactivated, respectively, with various types of molecular footprints in lung cancer cells (Fong et al., 1999; Zochbauer-Muller et al., 2002). Among the most notable tumor suppressor genes are p53, the cyclin-dependent kinase inhibitor p16 and the retinoblastoma protein (Rb). The mutation analysis of p53 is useful for diagnosis in lung cancer due to highly frequent, stable, and well-distributed point mutations occurring in exons 5 to 8 (Skaug et al., 2000). Codon 248 in exon 7 and codon 273 in exon 8 are two major mutation hot spots of p53 in human lung cancers (Behn and Schuermann, 1998; Wakamatsu et al., 1999; Jassem et al., 2001; Feng et al., 2003). The Rb-p16 cell cycle pathway has a crucial role in lung tumorigenesis. Impairment of the Rb-p16 pathway has been shown to occur in almost all lung tumors. Inactivating mutations and deletions of Rb and p16 are main mechanisms responsible for the alterations (Wikenheiser-Brokamp, 2006; Wikman and Kettunen, 2006). The epidermal growth factor receptor (EGFR) is a cell membrane receptor that plays a key role in non-small cell lung cancer (NSCLC) development and progression. Huang et al. (2004) and Paez et al. (2004) reported that mutations of EGFR are present in a subset of pulmonary adenocarcinomas and that tumors with EGFR mutations are highly sensitive to gefitinib, an EGFR kinase inhibitor. EGFR mutations are higher in Japanese than in Caucasian patients, which were correlated with the clinical responsiveness to gefitinib in NSCLC (Huang et al., 2004; Paez et al., 2004). Among these mutations of EGFR, the most frequently occurring single nucleotide substitution was at nucleotide 273 (T→G) in exon 21, resulting in substitution of arginine for leucine at position 858 (L858R) (Huang et al., 2004; Paez et al., 2004; Kosaka et al., 2004; Lynch et al., 2004).

Microsphere (bead)-based suspension array technology has been shown to offer a novel platform for high-throughput detection and has been utilized with increasing frequency (Ye et al., 2001; Iannone et al., 2003; Keyes et al., 2003; Dunbar and Jacobson, 2005; Jiang et al., 2006; Diaz et al., 2006; Dehqanzada et al., 2007). The most outstanding advantage of suspension array is that the accurate detection of the specimens could be achieved in a multiplex platform by mixing multiple probes in a single reaction (Nolan and Mandy, 2001; Kellar and Iannone, 2002; Nolan and Sklar, 2002). For DNA hybridization assays, the beads are internally labeled with a different ratio of red to infrared fluorophores allowing each bead to possess a unique spectral address. Biotinylated polymerase chain reaction (PCR) amplicon is allowed to hybridize with the bead coupled by specific probe, labeled with a fluorescent reporter molecule (streptavidin-R-phycoerythrin) and measured with the flow cytometer (Diaz and Fell, 2005; Diaz et al., 2006).

The purpose of this study was to develop a rapid and reliable detection strategy for molecular diagnosis of lung cancer. Here, the oligonucleotide probes were designed to detect the hot spot genomic DNA mutations of p53, p16, Rb, and EGFR in 125 lung cancer and 30 adjacent histologically tumor-free lung tissue samples. According to the promising results, we envision that this liquid-based method will be a useful diagnostic tool in clinical oncology.

MATERIAL AND METHODS

Patients and tissue samples

A total of 125 lung cancer specimens and 30 adjacent histologically tumor-free lung tissue samples were analyzed in this study. The cohort of the 125 tumor patients consisted of 37 small cell lung cancer (SCLC) and 88 NSCLC. According to the UICC recommendations, 45 patients were classified as stage I, 44 patients as stage II, and 36 patients as stage III. All patients were treated surgically between 1999 and 2008. At the time of primary resection, patients' median age was 57 years (range 14-74). The male:female ratio was 93/32.

DNA extraction and multiplex PCR

Genomic DNA was extracted from tumor tissue and tumor-free tissue using the DNeasy Blood & Tissue Kit (Qiagen, China). Specific PCR amplification primers were designed to amplify each target gene containing the hot spot mutation site, and the general primer pair with complement sequence to 5' segment of specific primers was 5'-biotin-labeled (Shanghai Sangon, China) (Table 1). Singleplex reactions were used during initial testing, and multiplex reactions were carried out in the final format of the assay. Singleplex reaction mixtures typically contained 1X Optimized Dynazyme buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTP, 10 pmoL specific primer pair, 2 U Dynazyme EXT DNA polymerase (New England Biolabs, China), 1 ng genomic DNA, and nuclease-free water for a final volume of 50 µL. Samples were PCR-amplified on a PTC-100 thermocycler (MJ Research, USA) with the following conditions: 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; a final 8-min extension at 72°C. Multiplex reaction mixtures typically contained 1X Optimized Dynazyme buffer (containing 1.5 mM $MgCl_2$), 0.2 mM dNTP, 2 pmoL each special primer pair, 20 pmol general primers, 3 U Dynazyme EXT DNA polymerase, 50 ng genomic DNA, and nuclease-free water for a final volume of 50 µL. Reactions were performed in a PTC-100 thermocycler with the following conditions: 94°C for 10 min; 10 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final 8-min extension at 72°C.

Table 1. Description of primers designed to amplify regions of target genes.

Primer	Description	Sequence (5'→3')
General F	General forward primer	Biotin-CGCGCGACTAGGGAAGACTTC
General R	General reverse primer	Biotin-AGGAAGACTTCCGAGCGGTC
p53-F7	p53 exon 7 forward	CGCGCGACTAGGGAAGACTTCGTTGGCTCTGACTGTACCA
p53-R7	p53 exon 7 reverse	AGGAAGACTTCCGAGCGGTCCTGGAGTCTTCCAGTGTGA
p53-F8	p53 exon 8 forward	CGCGCGACTAGGGAAGACTTCTGGTAATCTACTGGGACGG
p53-R8	p53 exon 8 reverse	AGGAAGACTTCCGAGCGGTCCTCGCTTAGTGCTCCCTGG
p16-F2	p16 exon 2 forward	CGCGCGACTAGGGAAGACTTCGTCATGATGATGGGCAGCGC
p16-R2	p16 exon 2 reverse	AGGAAGACTTCCGAGCGGTCCTGAGGGACCTTCCGCGGC
Rb-F20	Rb exon 20 forward	CGCGCGACTAGGGAAGACTTCTGTATCGGCTAGCCTATCTC
Rb-R20	Rb exon 20 reverse	AGGAAGACTTCCGAGCGGTCTTGGTCCAAATGCCTGTCTC
EGFR-F21	EGFR exon 21 forward	CGCGCGACTAGGGAAGACTTCGGCATGAACTACTTGGAGG
EGFR-R21	EGFR exon 21 reverse	AGGAAGACTTCCGAGCGGTCTTTGCCTCCTTCTGCATGG

Coupling of probes to microspheres (beads)

Five positive control (Pc) and 1 negative control (Nc) probes as well as 10 capture probes specific for each normal (No) and mutant (Mu) site of four target genes were designed and optimized for assay (Table 2). The target hot spot mutations detected are codon 248 in exon 7 and 273 in exon 8 of p53, codon 75 in exon 2 of p16, codon 675 in exon 20 of Rb and codon 858 in exon 21 of EGFR. Probes had Tm's higher than 55°C and GC contents around 50%. Probes were synthesized with a C-12 linker on the 5' end (Integrated DNA Technologies, USA) to reduce steric hindrance from beads (Diaz and Fell, 2004). Probes were covalently coupled to beads (Luminex, Inc., USA) by a carbodiimide coupling method (Diaz and Fell, 2004) with slight modifications. The coupled bead stock was vortexed and sonicated for 20 s, and 200 µL (5×10^6 beads) were transferred to a low-binding 1.5-mL microcentrifuge tube. The beads were pelleted by centrifugation at 8000 *g* for 3 min, and the supernatant was discarded. The beads were resuspended in 25 µL 0.1 M MES (2[N-morpholino] ethanesulfonic acid), pH 4.5, with vortex and sonication steps (20 s each). Probe (0.2 nmoL) was added and the solution was vortexed. A 1-µg/µL solution of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) (Pierce, USA) was freshly prepared, and 2.5 µL was added to the bead solution. The tubes were then vortexed, and incubated in the dark at room temperature for 30 min. The EDC addition and incubation steps were repeated once. The beads were collected, washed with 0.5 mL 0.02% Tween 20 followed by 0.5 mL 0.1% SDS, mixed and resuspended in 100 µL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and stored in the dark at 4°C before use.

Table 2. List of probes used for target gene identification.

Probe	Description	Sequence (5'→3')	Recognized codon	Reference
p53-7Pc	p53 positive control probe	tacaactacatgtgtaacagttcctgc	231~240	
p53-7-248R	p53 248 normal probe	ggcatgaaccggaggcccatcctcacc	245~253	Wakamatsu et al., 1999
p53-7-248L	P53 248 mutated probe	ggcatgaaccTgaggcccatcctcacc	245~253	
p53-8Pc	p53 positive control probe	cgcacagaggaagagaatctccgcaag	283~291	
p53-8-273R	p53 273 normal probe	agctttgaggtgcgtgtttgtgcctgt	269~277	Behn and Schuermann, 1998
p53-8-273H	P53 273 mutated probe	agctttgaggtgcAtgtttgtgcctgt	245~277	
p16-2Pc	p16 positive control probe	gctgggccatcgcgatgtcgcacggta	119~127	
p16-2-75H	p16 75 normal probe	cgccgaccccgccactctcacccg	71~78	Xiao et al., 1995
p16-2-75N	p16 75 mutated probe	cgccgaccccgcAactctcacccg	71~78	
Rb-20Pc	Rb positive control probe	ccgcagaatgagtatgaactcatgaga	688~696	
Rb-20-675E	Rb 675 normal probe	ctgagcacccagaattagaacatatc	670~680	Onadim et al., 1992
Rb-20-675STOP	Rb 675 mutated probe	ctgagcacccaTaattagaacatatc	670~680	
EGFR -21Pc	EGFR positive control probe	ttggaggaccgtcgcttggtgcaccgc	827~835	
EGFR -21-858L	EGFR 858 normal probe	acagattttgggctggccaaactg	854~861	Zhao et al., 2007; Jiang et al., 2005
EGFR -21-858R	EGFR 858 mutated probe	acagattttgggcGggccaaactg	854~861	
EGFR -21Nc	Negative control probe	ttggCggaccgtcgcttggtgcaccgc	827~835	

The underlined upper-case nucleotides in the Sequence column correspond to the mutated site.

Hybridization of coupled probes with PCR amplicons

The bead mixture was diluted in 1.5X TMAC (1.5 M tetramethyl ammonium

chloride, 75 mM Tris, 6 mM EDTA, and 0.15% sarkosyl, pH 8.0) hybridization buffer to a final concentration of 150 of each bead per microliter. A volume of 33 µL bead solution was mixed with 17 µL biotinylated amplicons in a well of a 96-well microtiter plate. The mixture was incubated in a thermal cycler for 5 min at 94°C and 30 min at 52°C, sequentially.

Labeling and measuring

The hybridized beads were pelleted by centrifugation at 2000 *g* for 3 min. The pellet was resuspended in 75 µL labeling buffer (containing 4 µg/mL phycoerythrin-conjugated streptavidin; Molecular Probes, China), incubated 10 min at 52°C in the thermal cycler and analyzed with the Luminex® 100™ (Luminex, Inc., USA). All reported median fluorescence intensity measurements (MFI) were background corrected (F-F0), where F0 was the background signal determined from the fluorescence measurement of a blank sample (TE buffer only). A positive Luminex response has been defined as twice the signal background, after the background has been subtracted (Diaz and Fell, 2005). The identification of mutation was defined as an No/Mu ratio (dividing the MFI of normal probe by that of mutated probe) less than 1.5. In addition, the reaction was considered to be valid when the Nc/Pc ratio (dividing the MFI of negative probe by that of positive probe) is less than 5%.

Sequencing of amplicons

Some of the specimens were verified by DNA sequencing. Amplification reaction was carried out as the singleplex reaction described above. Amplicons were purified by a standard method and sequenced directly by an ABI-Prism 377 sequencer, according to manufacturer instructions.

RESULTS

Specificity of the probes and sensitivity of the suspension microarray

Specific PCR primers were designed to amplify each mutation-containing region, and five No and Mu probe sets were designed to detect two mutations in p53 and one mutation in p16, Rb and EGFR. To test specificity of the probes and sensitivity of the suspension microarray, five genomic DNA samples with known genotypes were screened. Representative data from these experiments are shown in Figure 1. According to the cut-off value, samples 1 and 4 have one gene mutation in p53 and EGFR, respectively; samples 2 and 3 have two mutated genes (sample 2: p53 + p16; sample 3: Rb + EGFR), and sample 5 has three mutated genes (p53 + Rb + EGFR). The results from suspension microarray judged according to No/Mu ratio were consistent with expectations based on sequence data. From these results, it appears that No/Mu <1.5 can be considered valid for distinguishing the mutations.

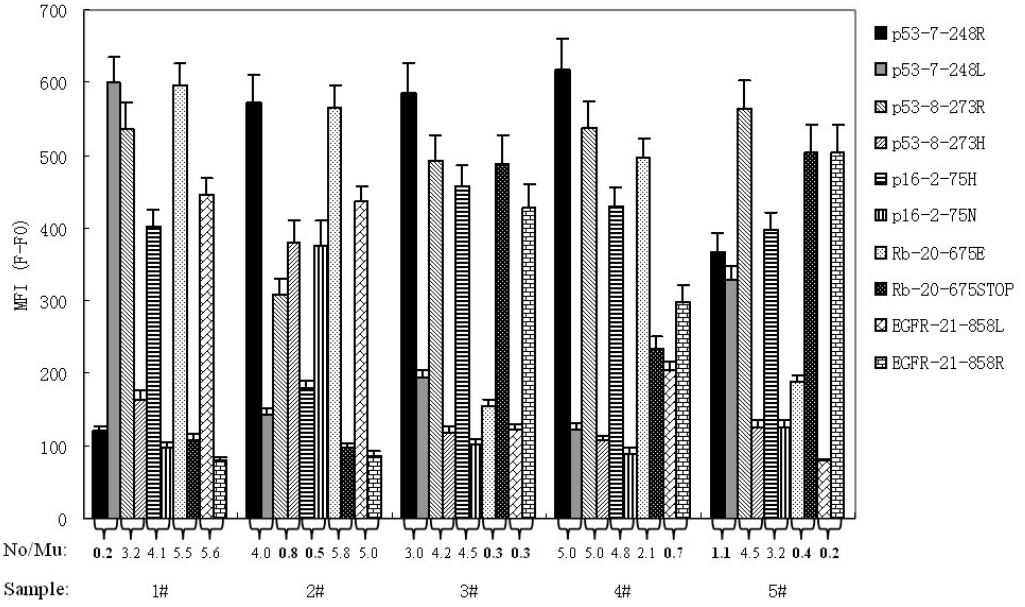

Figure 1. Luminex response to genomic DNA extracted from lung tumor specimens. Ten bead classifications, each coupled to one capture probe [normal (No) or mutated (Mu)], were hybridized to biotinylated PCR amplicons, labeled with streptavidin-phycoerythrin, and analyzed using the Luminex100. Background fluorescence (F0) from TE controls containing no PCR product was removed to yield values of median fluorescence intensity (MFI). Column height depicts the mean and bars the range for three replicate analyses. Representative results from 5 individuals are shown. The data of No/Mu below 1.5 were in bold and indicated mutation.

Detection panel of p53, p16, Rb, and EGFR for lung cancer diagnosis

As the Luminex assay worked as designed based on preliminary studies, 125 lung cancer specimens and 30 tumor-free controls were then tested. The Luminex response was strong for most samples, and similar sensitivity was observed for all specific probes in these samples. When used as a single marker for lung cancer diagnosis, p53, p16, Rb, or EGFR yielded low detection rates (11.2~36.8%) in lung cancer specimens. When integrated into a diagnostic panel, the coverage of tumor, which is based on detection of at least one of these mutations, was raised. p53 + EGFR had a higher sensitivity of 50.4% compared to other couples; p53 + p16 + EGFR was the most effective ternate group with a sensitivity of 60.0%, and detection of four markers elevated the sensitivity to 68.0% (Table 3). In the tumor-free group, only 3 cases were found to have one point mutation (p53, p16 and EGFR). Therefore, the specificity and the accuracy of four-marker detection were 90.0 and 72.3%, respectively. The mutation rates of the four-marker panel in stage I, stage II and stage III were 62.2% (28/45), 65.9% (29/44) and 75.0% (27/36), respectively. In 88 cases of NSCLC, the mutation rates of p16 and Rb were 19.3% (17 cases) and 2.3% (2 cases), while in 37 cases of SCLC, the mutation rates of p16 and Rb were 5.4% (2 cases) and 32.4% (12 cases). The distribution of p53 and EGFR mutation was not significantly correlated with tumor type.

Table 3. Diagnostic value of single or combined detection of p53, p16, Rb, and EGFR.

Target gene	Mutated cases	Sensitivity	Specificity	Accuracy
p53	47	36.8% (46/125)	96.7% (29/30)	48.4% (75/155)
p16	20	15.2% (19/125)	96.7% (29/30)	31.0% (48/155)
Rb	14	11.2% (14/125)	100% (30/30)	28.4% (44/155)
EGFR	24	18.4% (23/125)	96.7% (29/30)	33.5% (52/155)
p53 + p16	62	48.0% (60/125)	93.3% (28/30)	56.8% (88/155)
p53 + Rb	58	45.6% (57/125)	96.7% (29/30)	55.5% (86/155)
p53 + EGFR	65	50.4% (63/125)	93.3% (28/30)	58.7% (91/155)
p16 + Rb	34	26.4% (33/125)	96.7% (29/30)	40.0% (62/155)
p16 + EGFR	41	31.2% (39/125)	93.3% (28/30)	43.2% (67/155)
Rb + EGFR	36	28.0% (35/125)	96.7% (29/30)	41.3% (64/155)
p53 + p16 + Rb	74	57.6% (72/125)	93.3% (28/30)	64.5% (100/155)
p53 + p16 + EGFR	78	60.0% (75/125)	90.0% (27/30)	65.8% (102/155)
p53 + Rb + EGFR	75	58.4% (73/125)	93.3% (28/30)	65.2% (101/155)
p16 + Rb + EGFR	52	40.0% (50/125)	93.3% (28/30)	50.3% (78/155)
p53 + p16 + Rb + EGFR	88	68.0% (85/125)	90.0% (27/30)	72.3% (112/155)

Sensitivity: proportion of positive results among all lung cancer patients. Specificity: proportion of negative results among all tumor-free patients. Accuracy: true-positive and -negative results among all results of the test.

Luminex verification through sequencing of amplicons

Sequence analysis of singleplex amplicons generated from some specimens with individual primer pair was used to verify the Luminex assay. Sequencing results indicated that Luminex response could specifically identify the mutation in target genes (data not shown).

DISCUSSION

Abnormalities of p53, Rb, p16, and EGFR are frequent molecular events in lung cancer. However, nearly all studies of these biomarkers, examining the clinical associations and prognostic value of alterations, have been conducted with single biomarkers or dual combinations, such as Rb with p16 (Shapiro et al., 1995; Kratzke et al., 1996; Kashiwabara et al., 1998) and Rb with p53 (Dosaka-Akita et al., 1997). This is the first study that combined p53, Rb, p16, and EGFR in a multimarker panel for lung cancer diagnosis.

Successful treatment of lung cancer remains strongly dependent on identification of early-stage tumors. However, in some cases, the sample size is too small for multimarker pathological diagnosis. Although conventional solid microarray (chip) technologies would be very sensitive and specific for multimarker detection from a small quantity of specimen, chips are subject to the limitation of very small amounts of probe available and hybridization in a two-dimensional format. A two-dimensional format leads to reading difficulties, inadequate exposure of components to reagents, and concentration gradients across the two-dimensional surface. Besides, chips are restricted to one sample per slide, making them less cost-effective. In comparison, the suspension-based Luminex® 100™ MASA™ system has several advantages. First, the three-dimensional surface area of the beads provides a higher concentration of probes available for hybridization. Second, hundreds of beads coupled with each probe can be scanned in a single reaction, which produces digital data with statistical significance. Third, the bead-based system is user-friendly, simple in design and easy to perform, and data are automatically analyzed and calculated. Moreover, the assay is inexpensive

to perform (Wallace et al., 2005; Wilson et al., 2005). Studies have demonstrated the simultaneous application of four to fifteen probes in the Luminex system without compromising the sensitivity or specificity of the test (Dunbar et al., 2003; Diaz and Fell, 2004). Here, using the Luminex system, we demonstrated its capacity to detect five point mutations in four genes, simultaneously. This method needed only a small quantity of specimen and allowed a sample to be processed in less than 1 h post-PCR amplification. Statistical data for each specimen were acquired within 30 s, which were automatically converted to MFI values by the Luminex system software.

In our analysis of clinical specimens using Luminex system, the abnormalities of p53, Rb, p16, and EGFR are consistent with previously published data, which emphasized the importance of these tumor suppressor genes and oncogene in lung tumorigenesis. Sixty-eight percent of lung cancer specimens have at least one mutation in the four tested genes, which revealed that detection with the four-gene panel is competent for molecular diagnosis of lung cancer. Besides, the frequency of mutation was found to be inconsistent for these markers. The abnormality rate of p53 was about 20% higher than that of p16, Rb and EGFR, which suggested that the genetic alteration of p53 was the most significantly associated with lung cancer. The data of Luminex also revealed that simultaneous detection of two, three and four markers elevated the sensitivity of detecting mutation to 50.4, 60.0 and 68.0%, respectively. The simultaneous detection of the four markers had a sensitivity 30~60% higher than these markers had achieved separately, and the specificity and accuracy remained at a high level of 90.0 and 72.3%. Therefore, a panel of four markers provided a more reliable and informative approach than a single-gene assay in the detection of lung cancer. Moreover, the mutation rate of this multimarker panel remained very high for lung cancer patients at early pathological stages, e.g., 62.2% for patients at stage I and 65.9% for patients at stage II. This indicated that the panel of p53, Rb, p16, and EGFR has a great potential for clinical application in early detection of lung cancer. In addition, the distribution of mutations of p16 and Rb was preferential in different subtypes of lung cancer, i.e., p16 mutation occurred more frequently in NSCLC than in SCLC (19.3 versus 5.4%), while the Rb mutation mainly distributed in SCLC (32.4 versus 2.3%). Therefore, the mutation detection of p16 and Rb may serve not only as an early diagnostic basis for lung cancer, but also as a means for differentiation of NSCLC from SCLC. Despite that high-throughput detection of mutations of p53, p16, Rb, and EGFR has been shown to have satisfactory sensitivity and specificity for lung cancer diagnosis, the molecular diagnosis of cancer still requires that it be combined with complete history taking, thorough physical examination and proper imaging study.

In conclusion, we describe a sensitive, specific and rapid PCR-based, bead-probe liquid array system for the simultaneous detection of mutations of p53, Rb, p16, and EGFR in genomic DNA extracted directly from clinical materials. Although the mutant sites that we detected in this study account for only a limited subset of all hot spot mutations, the mutation rates of these markers were comparatively high at early pathological stages in lung cancers, indicating that combined detection of p53, Rb, p16, EGFR, and other tumor suppressor genes may have a great potential for early diagnosis of lung cancer.

ACKNOWLEDGMENTS

Research supported by the Ministry of Science and Technology of China (grants #96-906-01-18, #2001BA703B11), Social Development Project of Science and Technology Department of Jiangsu Province (grant #BS2006005).

REFERENCES

Behn M and Schuermann M (1998). Sensitive detection of p53 gene mutations by a 'mutant enriched' PCR-SSCP technique. *Nucleic Acids Res.* 26: 1356-1358.

Dehqanzada ZA, Storrer CE, Hueman MT, Foley RJ, et al. (2007). Assessing serum cytokine profiles in breast cancer patients receiving a HER2/neu vaccine using Luminex technology. *Oncol. Rep.* 17: 687-694.

Diaz MR and Fell JW (2004). High-throughput detection of pathogenic yeasts of the genus trichosporon. *J. Clin. Microbiol.* 42: 3696-3706.

Diaz MR and Fell JW (2005). Use of a suspension array for rapid identification of the varieties and genotypes of the *Cryptococcus neoformans* species complex. *J. Clin. Microbiol.* 43: 3662-3672.

Diaz MR, Boekhout T, Theelen B, Bovers M, et al. (2006). Microcoding and flow cytometry as a high-throughput fungal identification system for *Malassezia* species. *J. Med. Microbiol.* 55: 1197-1209.

Dosaka-Akita H, Hu SX, Fujino M, Harada M, et al. (1997). Altered retinoblastoma protein expression in nonsmall cell lung cancer: its synergistic effects with altered ras and p53 protein status on prognosis. *Cancer* 79: 1329-1337.

Dunbar SA and Jacobson JW (2005). Rapid screening for 31 mutations and polymorphisms in the cystic fibrosis transmembrane conductance regulator gene by Luminex xMAP suspension array. *Methods Mol. Med.* 114: 147-171.

Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, et al. (2003). Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. *J. Microbiol. Methods* 53: 245-252.

Feng Z, Hu W, Rom WN, Costa M, et al. (2003). Chromium(VI) exposure enhances polycyclic aromatic hydrocarbon-DNA binding at the p53 gene in human lung cells. *Carcinogenesis* 24: 771-778.

Fong KM, Sekido Y and Minna JD (1999). Molecular pathogenesis of lung cancer. *J. Thorac. Cardiovasc. Surg.* 118: 1136-1152.

Huang SF, Liu HP, Li LH, Ku YC, et al. (2004). High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. *Clin. Cancer Res.* 10: 8195-8203.

Iannone MA, Taylor JD, Chen J, Li MS, et al. (2003). Microsphere-based single nucleotide polymorphism genotyping. *Methods Mol. Biol.* 226: 123-134.

Jassem E, Niklinski J, Rosell R, Niklinska W, et al. (2001). Types and localisation of p53 gene mutations: a report on 332 non-small cell lung cancer patients. *Lung Cancer* 34 (Suppl 2): S47-S51.

Jiang B, Zhu GS, Liu F, Gong SJ, et al. (2005). Mutational analysis of EGFR in Chinese patients with NSCLCs. *Acad. J. Sha. Sec. Med. Univ.* 25: 1148-1150.

Jiang HL, Zhu HH, Zhou LF, Chen F, et al. (2006). Genotyping of human papillomavirus in cervical lesions by L1 consensus PCR and the Luminex xMAP system. *J. Med. Microbiol.* 55: 715-720.

Kashiwabara K, Oyama T, Sano T, Fukuda T, et al. (1998). Correlation between methylation status of the p16/CDKN2 gene and the expression of p16 and Rb proteins in primary non-small cell lung cancers. *Int. J. Cancer* 79: 215-220.

Kellar KL and Iannone MA (2002). Multiplexed microsphere-based flow cytometric assays. *Exp. Hematol.* 30: 1227-1237.

Keyes KA, Mann L, Cox K, Treadway P, et al. (2003). Circulating angiogenic growth factor levels in mice bearing human tumors using Luminex Multiplex technology. *Cancer Chemother. Pharmacol.* 51: 321-327.

Kosaka T, Yatabe Y, Endoh H, Kuwano H, et al. (2004). Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res.* 64: 8919-8923.

Kratzke RA, Greatens TM, Rubins JB, Maddaus MA, et al. (1996). Rb and p16INK4a expression in resected non-small cell lung tumors. *Cancer Res.* 56: 3415-3420.

Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 350: 2129-2139.

Nolan JP and Mandy FF (2001). Suspension array technology: new tools for gene and protein analysis. *Cell Mol. Biol.* 47: 1241-1256.

Nolan JP and Sklar LA (2002). Suspension array technology: evolution of the flat-array paradigm. *Trends Biotechnol.* 20: 9-12.

Onadim Z, Hogg A, Baird PN and Cowell JK (1992). Oncogenic point mutations in exon 20 of the RB1 gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype. *Proc. Natl. Acad. Sci. U. S. A.* 89: 6177-6181.

Paez JG, Janne PA, Lee JC, Tracy S, et al. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500.

Shapiro GI, Edwards CD, Kobzik L, Godleski J, et al. (1995). Reciprocal Rb inactivation and p16INK4 expression in primary lung cancers and cell lines. *Cancer Res.* 55: 505-509.

Skaug V, Ryberg D, Kure EH, Arab MO, et al. (2000). p53 mutations in defined structural and functional domains are

related to poor clinical outcome in non-small cell lung cancer patients. *Clin. Cancer Res.* 6: 1031-1037.
Wakamatsu K, Nakanishi Y, Takayama K, Miyazaki H, et al. (1999). Frequent expression of p53 protein without mutation in the atypical epithelium of human bronchus. *Am. J. Respir. Cell Mol. Biol.* 21: 209-215.
Wallace J, Woda BA and Pihan G (2005). Facile, comprehensive, high-throughput genotyping of human genital papillomaviruses using spectrally addressable liquid bead microarrays. *J. Mol. Diagn.* 7: 72-80.
Wikenheiser-Brokamp KA (2006). Retinoblastoma regulatory pathway in lung cancer. *Curr. Mol. Med.* 6: 783-793.
Wikman H and Kettunen E (2006). Regulation of the G1/S phase of the cell cycle and alterations in the RB pathway in human lung cancer. *Expert. Rev. Anticancer Ther.* 6: 515-530.
Wilson WJ, Erler AM, Nasarabadi SL, Skowronski EW, et al. (2005). A multiplexed PCR-coupled liquid bead array for the simultaneous detection of four biothreat agents. *Mol. Cell Probes* 19: 137-144.
Xiao S, Li D, Corson JM, Vijg J, et al. (1995). Codeletion of p15 and p16 genes in primary non-small cell lung carcinoma. *Cancer Res.* 55: 2968-2971.
Ye F, Li MS, Taylor JD, Nguyen Q, et al. (2001). Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum. Mutat.* 17: 305-316.
Zhao YL, Li Q, Li XH, Han WD, et al. (2007). Epidermal growth factor receptor mutation in non-small cell lung cancer and breast cancer. *Yi. Chuan* 29: 547-553.
Zochbauer-Muller S, Gazdar AF and Minna JD (2002). Molecular pathogenesis of lung cancer. *Annu. Rev. Physiol.* 64: 681-708.