High efficiency for activated *KRAS* detection from peripheral blood using weighted enzymatic gene chip array method

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Abstract. The *KRAS* mutation test is recommended that it may determine which patients with advanced colorectal or lung cancer are eligible for anti-EGFR targeted therapies. Traditional molecular techniques such as polymerase chain reaction (PCR) combined direct sequencing are limited by the difficulty of obtaining patients' cancer tissues for analysis. Finding an alternative method to detect *KRAS* gene mutation is important. This study aims to estimate the efficiency of Activating *KRAS* Detection Chip using a weighted enzymatic chip array (WEnCA) platform, which was successfully established in our previous study, in detecting activated *KRAS* mutations from the peripheral blood of cancer patients. The paired tumor tissue and peripheral blood specimens were collected from 377 patients with colorectal or non-small cell lung cancer. DNA were extracted from tissue samples and applied to detect *KRAS* mutation using direct sequencing. RNA were extracted from peripheral blood samples and applied to detect the activated *KRAS* mutation using WEnCA. Results showed 122 tumor specimens with *KRAS* mutation, of which 112 were positive through WEnCA. The sensitivity, specificity and accuracy of WEnCA analysis for detecting activated *KRAS* mutation in peripheral blood were 91.8%, 94.5% and 93.6%, respectively. This study demonstrated that WEnCA is a sensitive, easier interpretation, and a convenient technique for detecting the circulating KRAS mutant from the peripheral blood of cancer patients.

Keywords: Colorectal cancer, Lung cancer, Peripheral blood, Weighted enzymatic chip array (WEnCA), Activating *KRAS* Detection Chip

1 Introduction

KRAS oncogene was one of the earliest discoveries of genetic alterations in colorectal and lung cancer. In recent years, *KRAS* mutations had been reported that are highly specific predictors of response to an-

ti-EGFR antibodies in metastatic colorectal cancer (mCRC) or to EGFR TKIs monotherapy in advanced non-small-cell lung cancer (NSCLC) [1-5]. Up to now, most using techniques for *KRAS* mutation detection like polymerase chain reaction (PCR) combining direct sequencing, fluorescent in situ hybridization (FISH) or real-time PCR are insensitive and time-consuming; moreover, the difficulty in cancer tissue specimen collection causes the limitation for these methods. Thus, an easier and more accurate method is quite important.

Recent studies have suggested that detecting the molecular markers of circulating tumor cells (CTCs) in peripheral blood of cancer patients has great potential for cancer diagnosis, prognosis and follow-up assessments [6]. Besides, combination of multiple biomarkers can improve the diagnosis in patients [7, 8]. In previous studies, we successfully constructed the Activating *KRAS* Detection Chip for detecting *KRAS* activation from peripheral blood, and demonstrated a weighted enzymatic chip array (WEnCA) platform to make the chip results more sensitive, accurate, and easier to read [9]. The target genes on the Activating *KRAS* Detection Chip are given different weighted scores based on the performance of each gene after *KRAS* activation. In this study, we further investigate the correlation between Activating *KRAS* Detection Chip with WEnCA platform and standard sequencing method for the detection of activated KRAS oncogene in peripheral blood and tumor tissues of cancer patients respectively.

2 Materials & Methods

2.1 Sample collection

Tumor tissues and corresponding peripheral blood samples of 377 randomized NSCLC and CRC patients were collected from Fooyin University Hospital and Kaohsiung Medical University Hospital. Sample obtainment and usage were approved by the Institutional Review Boards of the two hospitals.

2.2 DNA extraction and direct sequencing of KRAS gene

Genomic DNA was isolated from frozen cancerous tissues using proteinase-K (Stratagene, La Jolla, CA) digestion and phenol/chloroform

extraction procedure [10]. PCR analysis was performed to identify KRAS gene mutations in tumor tissues using designed sequences of oligonucleotide primers for KRAS exons 1 and 2 (**Table 1**). The PCR products were purified by a QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA) and then applied to direct sequencing using an automatic sequencer (Model 4200; LI-COR Inc., Lincoln, NE).

2.3 Total RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from collected peripheral blood specimens using QIAamp RNA Blood Mini Kit (Qiagen Inc., Valencia, CA). First strand cDNA was synthesized from total RNA using a reverse-transcription (RT)-PCR kit (Promega Corp., Madison, WI). The reaction mixtures were incubated at 42℃ for 2 h, heated to 95℃ for 5 min, and then stored at -80℃ until analysis.

2.4 Weighted enzymatic chip array (WEnCA)

The design and preparation of Activating *KRAS* Detection Chip was based on our previous study [11]. OMP3 (Oligonucleotide Modeling Platform, DNA Software, Ann Arbor, MN) was used to design the probe sequences for target genes (**Table 2**). The newly synthesized oligonucleotide fragments were dissolved in deionized water and then applied to a Biojet Plus 3000 nanoliter dispensing system (BioDot Inc., Irvine, CA), which blotted the 22 target genes, 1 tuberculosis gene, and 1 housekeeping gene (β-actin) on a Nytran SuperCharge nylon membrane (Schleicher and Schuell) in triplicate (**Figure 1A**). Dimethyl sulfoxide (DMSO) was also dispensed onto the membrane as a blank control. The membrane was completely prepared after cross-linking procedure. The expressions level of each gene spot measured on Activating *KRAS* Detection Chip were quantitated, and then normalized with reference gene (β-actin) density. When the normalized spot density was 2 or more, it is defined as the overexpressed gene spot. Each overexpressed gene was then multiplied by weighted value ranging from 1 to 4 according to its performance after *KRAS* activation. The chip was defined as positive result while the total score is 20 or more (**Figure 1B**).

2.5 Preparation of digoxigenin-labeled cDNA targets and hybridization

First-strand cDNA targets were applied to biotin labeling, and the biotin-labeled probes then hybridized with the Activating *KRAS* Detection Chip. The hybridized chip followed washing, blocking and color development procedures using a GeneCling® Enzymatic Gene Chip Detection Kit ((MedicoGene Biotechnology Corp., Los Angel, CA). The hybridized chips were then scanned using an Epson Perfection 1670 flatbed scanner (SElKO EPSON Corp., Nagano-ken, Japan). Subsequent quantification analysis of each spot's intensity was carried out using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA). The interpretation of gene density and chip result are mentioned previously.

2.6 Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences Version 12.0 software (SPSS Inc., Chicago, IL). The correlation between the WEnCA and direct sequencing for the detection of KRAS oncogene in peripheral blood and tumor tissues was compared using the chi-square test. A P-value < 0.05 was considered statistically significant.

3 Results

We collected 377 peripheral blood samples and paired tumor tissue of NSCLC patients and CRC patients to evaluate clinical *KRAS* activation using Activating *KRAS* Detection Chip with WEnCA platform. Among 122 tissue samples with mutant *KRAS* through direct sequencing, activating *KRAS* were detected in 112 blood samples through WEnCA analysis (as positive *KRAS* chip result). On the other 255 tissues with wild-type *KRAS*, 241 blood samples were observed as negative *KRAS* chip result. The sensitivity, specificity, and accuracy of WEnCA analysis for detecting activated *KRAS* mutation in peripheral blood were 91.8%, 94.5% and 93.6%, respectively. The results demonstrated a significantly high correlation between Activating *KRAS* Detection Chip and standard direct sequencing method for the detection of activated KRAS mutation in peripheral blood and tumor tissues ($P< 0.05$) (**Table 3**).

4 Discussion

As single marker detection tests such as PCR combined direct sequencing are limited by the obtaining of tumor tissue suitable for molecular analysis, an alternative method for detecting *KRAS* gene mutation is needed. Since the Activating *KRAS* Detection Chip detects the expression of multiple *KRAS* downstream genes, it could show the entire situation of activated *KRAS* rather than detecting the status from single marker which may be undetectable because of the detection limitation. Besides, *KRAS* gene had been reported that having many mutation sites, including codon 12, 13, 15, 18, 31, but not all mutations can activate *KRAS*. In our earlier reports [9, 12], we found that blood samples with *KRAS* mutations in codon 31 showed negative results in chip assay. The reason may be that the mutation site of this codon cannot activate *KRAS*, and it also can explain the discordance of *KRAS* status between tumor and blood samples. To sum up, Activating *KRAS* Detection Chip with WEnCA platform can reflect the realer status of *KRAS* activation than PCR combined direct sequencing which detects mutations on certain codons.

Moreover, previous study reported the *KRAS* mutation in tumor may be undetected in the bloodstream and suggested that it may be caused due to the low DNA concentration other than the heterogeneity of primary cancer and metastatic lesions [13]. Our WEnCA platform which displays the overall status of *KRAS* activation could also overcome the heterogeneity issue. The results of this study demonstrated that detecting *KRAS* mutation in blood samples through WEnCA achieved higher sensitivity, specificity and accuracy than the direct sequencing for tumor tissues samples. This high-throughput assay is also an alternative diagnostic platform for patients who have the difficulty of obtaining tumor samples when compared with direct sequencing. It is suggested that detecting activated *KRAS* mutation in peripheral blood through WEnCA analysis is potential to serve as an assessment tool for cancer patients.

Table 1. Sequences of oligonucleotide primers used for PCR and sequencing of *KRAS*

	KRAS gene	
	Exon 1	Exon 2
Forward primer (5'→ 3')	TAATACGACTCACTATAGGGAGATATGTTGAGGGCCCATCTCTC	TAATACGACTCACTATAGGGTTCCTACAGGAAGCAAGTAG
Reverse primer (5'→ 3')	TCCTAGGTCAGCGCAACCAAAT	CACAAAGAAAGCCCTCCCCA
PCR product (bp)	131	148
Sequencing primer	CCCTATAGTGAGTCGTATTA	

Table 2. Oligonucleotide sequences of target genes on Activating KRAS Detection Chip

Gene	Oligonucleotide sequences (5'→ 3')
ATP2A2	ACCCGGACTTTGAAGGCTTGGATTGTGCAATCTTTGAATCCCCATACCCG
ATP6V0B	CATCGGCCATCGGAACTACCATGCAGGCTACTCCATGTTTGGGGCT
BCL2	ACAACATCGCCCTGTGGATGACTGAGTACCTGAACCGGCACCTGCACA
CALM2	GAAGCATTCCGTGTGTTTGATAAGGATGGCAATGGCTATATTAGTGCTGCAGAACTTCG
CEBPB	CCGCCTGCCTTTAAATCCATGGAAGTGGCCAACTTCTACTACGAGGCGGA
CLSTN1	TCTCTCCTCTCTCCTCCCCAGAGCACCCCCTGCCATCAGGGGGGTTGAAA
COL4A1	GCAAATGTGACTGCCATGGAGTGAAGGGACAAAAGGGTGAAAGAGGCCTC
CXCL11	GTTCAAGGCTTCCCCATGTTCAAAAGAGGACGCTGTCTTTGCATAGGCCC
CXCR4	CCCCATCCTCTATGCTTTCCTTGGAGCCAAATTTAAAACCTCTGCCCAGCAC
CYR61	CAGCAGCCTGAAAAAGGGCAAGAAATGCAGCAAGACCAAGAAATCCCCCG
DVL3	CGTCACCTTGGCGGACTTTAAGGGCGTTTTTGCAGCGACCCAGCTATAAGT
E2F4	TGAGATCACAGTGAGTGGCGGCCCTGGGACTGATAGCAAGGACAGT
ETS1	TGGAGCAGCCAGTCATCTTTCAACAGCCTGCAGCGTGTTCCCTCCTATGA
H2AFZ	CGTGGAGATGAAGAATTGGATTCTCTCATCAAGGCTACAATTGCTGGTGGTGGTGTC
L1CAM	CCTTCCTGGTGGTGTCCAACACGTCCACCTTCGTGCCCTATGAGATCAAA
LRP1	ATGCCTGTGAAAACGACCAGTATGGGAAGCCGGGTGGCTGCTCTGACAT
RAP1B	GGAAGATGAAAGAGTTGTAGGGAAGGAACAAGGTCAAAATCTAGCAAGACAATGGAACAACTGTG
RPL30	GCTCCAACTCGTTATGAAAAGTGGGAAGTACGTCCTGGGGTACAAGCAGAC
SLC25A5	TCTGATGGGATTAAGGGCCTGTACCAAGGCTTTAACGTGTCTGTGCAGGG
SPP1	GTGGACAGCCAGGACTCCATTGACTCGAACGACTCTGATGATGTAGATGAC
TAF12	CAGCACCCCTCCACAAGGCTCCATGGCCAATAGTACTGCAGTGGTAAAGA
TBX19	TCATCTGCTCAATGTGGTGGAGAGTGAGCTTCAGGCAGGGAGGGAAAAAG
β-actin	TGCATTGTTACAGGAAGTCCCTTGCCATCCTAAAAGCCACCCCACTTCTCTCTAAGGAGA

Table 3. Correlation between WEnCA and standard sequencing method results

	KRAS in tumor tissues (Sequencing)		p value
	Mutation (N=122)	Wild-type (N=255)	
KRAS in peripheral blood (WEnCA)			
Positive (N=126)	112	14	< 0.0001
Negative (N=251)	10	241	

* Sensitivity: 91.8%; Specificity: 94.51%; Accuracy: 93.63%; PPV (positive predictive value); 88.89%; NPV (negative predictive value): 96.02%.

(A)

ATP2A2	ATP6V0B	BMPR2	CALM2	CEBPB	CLSTN1	COL4A1	CXCL11	CXCR4
ATP2A2	ATP6V0B	BMPR2	CALM2	CEBPB	CLSTN1	COL4A1	CXCL11	CXCR4
ATP2A2	ATP6V0B	BMPR2	CALM2	CEBPB	CLSTN1	COL4A1	CXCL11	CXCR4
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
CYR61	DVL3	E2F4	ETS1	H2AFZ	L1CAM	LRP1	RAP1B	RPL30
SLC25A5	SPP1	TAF12	TBX19	blank	blank	blank	TB gene	B-actin
SLC25A5	SPP1	TAF12	TBX19	blank	blank	blank	TB gene	B-actin
SLC25A5	SPP1	TAF12	TBX19	blank	blank	blank	TB gene	B-actin

(B)

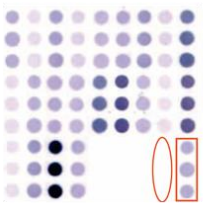
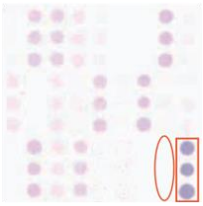
Chip Image		
Result	Positive (>20)	Negative (≦20)

Fig. 1. Schematic and Chip images of Activating KRAS Detection Chip by weighted enzymatic chip array (WEnCA) method

(A) A triplicated set of 22 mRNA markers for *KRAS* gene mutation was blotted on nylon membrane. β-actin and TB gene serving as positive and negative controls. (B) Positive result showed detectable activating KRAS mutation in peripheral blood, and the cutoff value was more than 20. On the contrary, negative result meant undetectable KRAS mutation in blood sample.

5 References

1. Linardou, H., Dahabreh, I.J., Kanaloupiti, D., Siannis, F., Bafaloukos, D., Kosmidis, P., Papadimitriou, C.A., and Murray, S.: 'Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer', Lancet Oncol, 2008, 9, (10), 962-972
2. Perez-Soler, R.: 'Erlotinib: recent clinical results and ongoing studies in non small cell lung cancer', Clinical cancer research : an official journal of the American Association for Cancer Research, 2007, 13, (15 Pt 2), s4589-4592
3. Sequist, L.V., Bell, D.W., Lynch, T.J., and Haber, D.A.: 'Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer', Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2007, 25, (5), 587-595
4. Jimeno, A., Messersmith, W.A., Hirsch, F.R., Franklin, W.A., and Eckhardt, S.G.: 'KRAS mutations and sensitivity to epidermal growth factor receptor inhibitors in colorectal cancer: practical application of patient selection', J Clin Oncol, 2009, 27, (7), 1130-1136
5. Lievre, A., Bachet, J.B., Boige, V., Cayre, A., Le Corre, D., Buc, E., Ychou, M., Bouche, O., Landi, B., Louvet, C., Andre, T., Bibeau, F., Diebold, M.D., Rougier, P., Ducreux, M., Tomasic, G., Emile, J.F., Penault-Llorca, F., and Laurent-Puig, P.: 'KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab', J Clin Oncol, 2008, 26, (3), 374-379
6. Young, R., Pailler, E., Billiot, F., Drusch, F., Barthelemy, A., Oulhen, M., Besse, B., Soria, J.C., Farace, F., and Vielh, P.: 'Circulating tumor cells in lung cancer', Acta cytologica, 2012, 56, (6), 655-660
7. Wang, J.Y., Lin, S.R., Wu, D.C., Lu, C.Y., Yu, F.J., Hsieh, J.S., Cheng, T.L., Koay, L.B., and Uen, Y.H.: 'Multiple molecular markers as predictors of colorectal cancer in patients with normal perioperative serum carcinoembryonic antigen levels', Clinical cancer research : an official journal of the American Association

for Cancer Research, 2007, 13, (8), 2406-2413
8. Tung, T.C., Lin, S.R., Wang, J.Y., and Chung, F.Y.: 'The use of multiple molecular markers as predictors of the clinical prognosis of patients with colorectal cancer', Genomic Medicine, Biomarkers, and Health Sciences, 2012, 4, (1), 30-33
9. Chen, Y.F., Wang, J.Y., Wu, C.H., Chen, F.M., Cheng, T.L., and Lin, S.R.: 'Detection of circulating cancer cells with K-ras oncogene using membrane array', Cancer Lett, 2005, 229, (1), 115-122
10. 'Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.', 1989, 6.22-26.34
11. Wang, J.Y., Yeh, C.S., Chen, Y.F., Wu, C.H., Hsieh, J.S., Huang, T.J., Huang, S.Y., and Lin, S.R.: 'Development and evaluation of a colorimetric membrane-array method for the detection of circulating tumor cells in the peripheral blood of Taiwanese patients with colorectal cancer', Int J Mol Med, 2006, 17, (5), 737-747
12. Chong, I.W., Chang, M.Y., Sheu, C.C., Wang, C.Y., Hwang, J.J., Huang, M.S., and Lin, S.R.: 'Detection of activated K-ras in non-small cell lung cancer by membrane array: a comparison with direct sequencing', Oncology reports, 2007, 18, (1), 17-24
13. Gutierrez, C., Rodriguez, J., Patino-Garcia, A., Garcia-Foncillas, J., and Salgado, J.: 'mutational status analysis of peripheral blood isolated circulating tumor cells in metastatic colorectal patients', Oncology letters, 2013, 6, (5), 1343-1345