EGFR AND *K-RAS* IN MOLECULARLY TARGETED THERAPY: FROM *IN SILICO* TO *IN VITRO* STUDY

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(Received: 23/01/2015; Revised: 26/01/2015; Accepted: 03/02/2015)

ABSTRACT

Molecularly targeted therapy is a new type of cancer treatment which uses monoclonal-antibodies or small substances to identify and attack cancer cells, except normal cell. This has more advantages than classical treatments. EGFR (Epidermal Growth Factor Receptor) and K-ras (Kirsten-ras) are considered as two well molecular-targeted agent.

In present study, we performed a systematic literature review in NCBI and computed average frequencies of EGFR and K-ras mutations in some common cancers. According to data analysis, we conclude that global published mutations which belong to these genes neither exclude each other nor associate with factors of race. We successfully designed and evaluated (1) primers for detection of EGFR and K-ras mutations by PCR-sequencing; (2) primer/probe sets are used for detection of the most seven common K-ras mutations by Allele-Specific-Real-time PCR (AS-Real-time PCR) as well as Allele-Specific PCR. Due to initial experimental results, an appropriate DNA extraction procedure from FFPE (Formalin-Fixed, Paraffin-Embedded) samples and optimal T_m for each primer couple of amplified mutation, containing regions on K-ras have been drawn. Results of K-ras mutation on colorectal cancer patients obtained from Tay Ninh Province, Viet Nam was firstly detected by using PCR-sequencing methods, then, confirmed by AS-Real-Time PCR.

Keywords: *EGFR, K-RAS, colorectal cancer, PCR-sequencing, Allele-Specific Real-time PCR.*

1. Introduction

Targeted therapy is a new progressive technology in the field of internal cancer treatment; it is relied on the application of research achievements in molecular biology (Sawyers C, 2004). This new treatment has more advantages. The drugs, which are used in this therapy, tend to be less toxic and site effects to non-cancerous cells while cancer cells are treated. Due to this intervene into special molecules which are directly relevant to tumorigenesis mechanism and proliferation of tumors, this molecular is called "molecular targets" and this therapy is called "molecularly targeted therapies" or "targeted therapies".

One of "molecular target", the first target for this therapy, is *EGFR* (Epidermal Growth Factor Receptor). It is glycoprotein, the cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands. Its function is tyrosine kinase, which exists on cell signaling and proliferation (Francoual *et al*, 2006). It is found that overexpression of *EGFR* is observed in many cancers (Nicholson *et al*, 2001). For this reason, the new therapy focuses on inhibiting the active of protein *EGFR* (Mendelsohn, 2002). Nowadays, two group of medicines effect on the target of *EGFR* are Tyrosine Kinase Inhibitors (TKIs), for examples: Erlotinib (Tarceva, Roche), and

monoclonal antibodies such as cetiximad (ErbituxTM, Bristol Myers Squibb and Merck KgaA), Pantitumumab (VectibixTM, Amgen) (Mendelsohn, 2002).

The basic for selection of personalized drug is screening of mutations on *EGFR* gene. If mutations, especially in the translated regions for *EGFR* gene's tyrosine kinase, are detected, TKIs will be used for treatment (Blanke, 2005, Tsao *et al*, 2005). Other no-mutated cases will be directly instructed in using antibodies resist *EGFR* such as Cetuximab hoặc Panitumumab (Mendelsohn, 2002).

Alternatively, the signal transduction of *EGFR* is RAS/RAF/MAPK pathway in which *K-ras* is important protein of this axis. (Karapetis *et al*, 2008). The activity of normal *K-ras* is depended on activity of *EGFR* receptor. However, *K-ras* protein is changed to be *EGFR*-independent if there are mutations on *K-ras* gene (The signal for proliferation and differentiation of cells also continuously transmitted) (Karapetis *et al*, 2008). Besides, patients are directly instructed to take the treatment with Catuximab and Panitumumab antibodies, it is necessary to have a screening on the mutation on *K-ras* gene whether or not. The reason is these antibodies only have affect on patient without mutations on *K-ras* (Benvenuti *et al.*, 2007; Amado *et al.*, 2008; Karapetis *et al*., 2008; Jönsson *et al.*, 2009). In some common cancer such as colorectal cancer, lung cancer, breast cancer, esophageal cancer… It is reported that either *EGFR* or *K-ras* with their mutations led to the disruptions of gene functions (Kwak E.L, 2006; Yunxia Z, 2010)

To sum up, it is necessary to have more researches on *EGFR* and *K-ras* mutations such as frequent mutated regions, mutation types, methods and techniques for detecting… in order to have more applications on targeted therapies for cancers treatment in Vietnam. These also are the targets of our studies.

2. Materials and methods

Clinical samples collection

In present study, 19 colorectal cancer specimens were collected from Tay Ninh Hospital, Vietnam from January, 2007 to May, 2011. Samples were embedded in paraffin and kept in -30^{o}C before further used.

Data mining, primer designed and DNA isolation

Data was collected on Pubmed by using different key words in order to study types of mutations on two gene *EGFR* and *K-ras*. Then, weighted average frequency was calculated to determine prominent mutations. Simultaneously, ANOVA analysis was carried out to evaluate the correlation between mutations and racial factors.

EGFR and *K-ras* sequences were collected from Genbank (NCBI), and primers for PCR were designed to detect promient mutations of *EGFR* and *K-ras*. Primer parameters were evaluated by several online bioinformatics tools such as BLAST (http://blast.ncbi.nlm.nih.gov/), IDT analyzer (www.idtdna.com/analyzer/Applications/OligoAnalyzer/), Primer Express, Annhyb, BatchPrimer 3 (http://probes.pw.usda.gov/batchprimer3/).

DNA extraction was isolated by using Phenol/Chloroform method (Sacchi, 1987). Because samples were embedded in paraffin, it was necessary to disintegrate paraffin by using xylene solution.

PCR-sequencing and Allele-Specific Real-time PCR

The amplifications were done in a total volume of 15µl, reaction was subjected to initial incubation at 95^{o}C for 5 min, followed by 40 cycles at 95^{o}C for 30s, X^{o}C for 30s (X was the annealing temperature of corresponding with each primer) , 72^{o}C for 30s and 72^{o}C for 6 min for final incubation. PCR products were directly performed sequencing in Macrogen Co. (Korea).

3. Results and methods

Data mining

We systematically reviewed more than 40 studies which related to analysis of mutations on *EGFR* and *K-ras* of five types of

cancer. In our revision, we found that paraffin embedded tissues were used more commonly (60% on the studies of *EGFR* and 67.27% on *K-ras*) than fresh tissues and frozen tissues. About the technique for detecting mutations, Sanger sequencing method was more commonly used than (54.05% on *EGFR* and 60.29% on *K-ras*) another methods such as Real-time PCR, COLD (Co-amplification at lower denaturation temp) PCR. Moreover, besides the characteristic of method's sensitivity, we considered the main factor affected the sensitivity which was the proportion of cancer cells presented in samples. Hence, the percentage of mutant cells in sample must to be greater than 70%, in contract, the samples would be eliminated.

For the aim to have a general revision about mutations on *EGFR* and *K-ras*, the weighted average frequencies were calculated. As the results, we concluded that (1) *K-ras* gene existed 7 prominent mutations, which were G12D, G12V, G13D, G12S, G12C, G12A, G12R; (2) *EGFR* gene existed 2 mutations with high frequencies which were L858R on exon 21 and gene deletion E746 – A750 on exon 19; (3) there was rarely mutant *EGFR* and almost no sense on colorectal cancer.

According to statistical analysis, there were no significant differences among various local population when making a comparison on *K-ras* and *EGFR* mutations (Table 1). Furthermore, we considered that types of mutation on each gene did not exclude each other. This statement was similar to the study of Sun and *at el* (2011).

Table 1. Sample's statistic and parameter ANOVA on different types of prominent mutation

7 prominent mutations on *K-ras* of colorectal cancer							
Total sample with *K-ras* mutation	**Europe**	**American**	**Asia**	**F**	**P value**	**F crit**	**Comment**
1605	1052	158					
G12D	546	338	69	1.957671	0.234331	7.708647	No significant differences at α = 5%
G12V	358	234	38	2.845997	0.166877	7.708647	
G13D	311	217	30	3.067208	0.154773	7.708647	
G12C	137	100	6	4.100888	0.112845	7.708647	
G12S	108	73	5	4.299204	0.106812	7.708647	
G12A	90	59	3	4.417707	0.103439	7.708647	
G12R	20	7	0	4.858892	0.092211	7.708647	

2 prominent mutations on *EGFR* of non-small cell lung cancer								
Total sample with *EGFR* mutation	**Australia**	**Europe**	**America**	**Asia**	**F**	**P value**	**F crit**	**Comment**
75	39	133	311					
L858R	13	15	40	103	2.29202	0.180811	5.987378	No significant differences at α = 5%
E746-A750	29	1	18	93	2.685696	0.152365	5.987378	

In silico study and PCR-sequencing primer designed

As mention at the introduction, in the RAS/RAF/MAPK signal pathway, if the *K-ras* was mutant, K-ras protein is EGFR-independent activated. For the therapy using Catuximab and Panitumumab antibodies applied on colorectal cancer patient, it is necessary to detect there was no any mutation on *K-ras*, otherwises, (in the patient with mutant *K-ras),* the therapy effect was worst. Therefore, we foscused on the detection of *K-ras* mutation.

K-ras nucleotide sequences were collected from GenBank via accession number: NC_000012.11. *K-ras* gene locate at 12p12.1 and containe 4 exons. Primer sequences were showed in Table 2. Parameters of primer pairs such as melting temperature, length, GC-base pair ratios, Gibbs Free Energy (ΔG) for secondary structures (hairpin, self-dimer, and heterodimer and product's length (Table 2) were rather fine for PCR-sequencing.

Table 2. Primer sequences and parameters

	Primer sequence (5'-3')	L	Tm	(1)	(2)	(3)	P (bp)
K-ras	F: GCCTGCTGAAAATGACTGAATA	22	53.3	1.89	-3.14	-5.09	176
	R: CTGTATCAAAGAATGGTCCTGCAC	24	55.9	0.99	-7.05		

Gibbs free energy (kcal/mol) for hairpin loop (1); homodimer (2) and heterodimer (3) structure formations. L: Product's length; Tm: melting temperature. F: Forward primer; R: Reverse primer.

DNA extraction

DNA extract protocol was modified various factors (Data not shown). We summarized the protocol for isolating DNA from paraffin embedded tissues (Fig. 1.). According to the protocol showed in figure 1, during isolating DNA, notably, it was necessary to pay more attention in washing with ethanol, incubating samples with proteinase K.

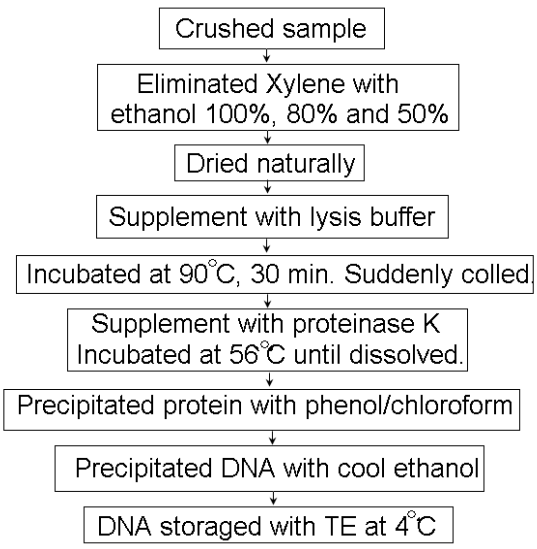


Figure 1. The protocol of DNA isolation

PCR-sequencing

In molecular, PCR sequencing is considered as the gold standard method. Therefore, at first, PCR-sequencing was applied in exon 1 region of *K-ras* on 19 colorectal cancer samples. As the results, we found out that there was not any mutation at codon 12, 13 on exon 1 of *K-ras* where concentrated 7 prominent mutation (Fig 2). However, some doubted-peak mutations were recognized showed in figure 3.

Figure 2. Results of sequencing codon 12, 13 on some sam

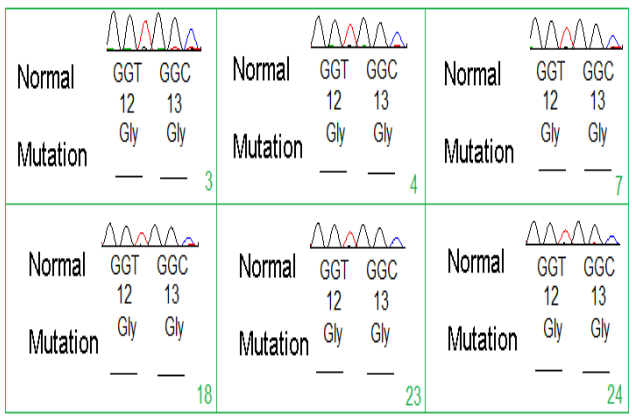
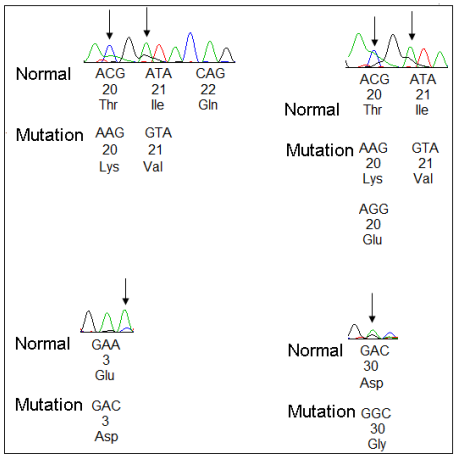


Figure 3. Some doubted-peak mutations (Arrow labeled)



According to results, the mutations on colorectal cancer patients at Tay Ninh Hospital (Vietnam) were so complex, various and no specific characteristic (Focus on codon 12, 13 of exon 1 of *K-ras*) as common reports. However, concerning to the PCR-sequencing technique, it was limited when carried out on those paraffin – embedded tissues, because we could not observe tissue samples slide on the lames under the light microscope to isolate the region where contained more than 70% of cancer cells in population. According to Varella Garcia *et al*., Sanger sequencing methods could not detect mutation of sample with the proportion of cancel cells under 25%. Moreover, samples used were adenocarcinoma, however, which was lack of clinical information such as invaded level, proportion of mutation cells on pathology, HE staining… Application of sequencing technique on those samples was so difficult.

Therefore, we tended to confirm the mutation located on codon 12, 13, which was detected via sequencing method, by another one. That is allele specific Real-time PCR method.

Allele-Specific Real-time PCR (ASR-PCR)

Primer designed was the most important step to successfully carry out the ASR-PCR to identify the mutation. The results of designing ASR-PCR primers were showed in table 3. Parameters of primer pairs such as melting temperature, length, GC-base pair ratios, Gibbs Free Energy (ΔG) for secondary structures (hairpin, self-dimer, and heterodimer and product's length were rather fine for PCR, theoretically.

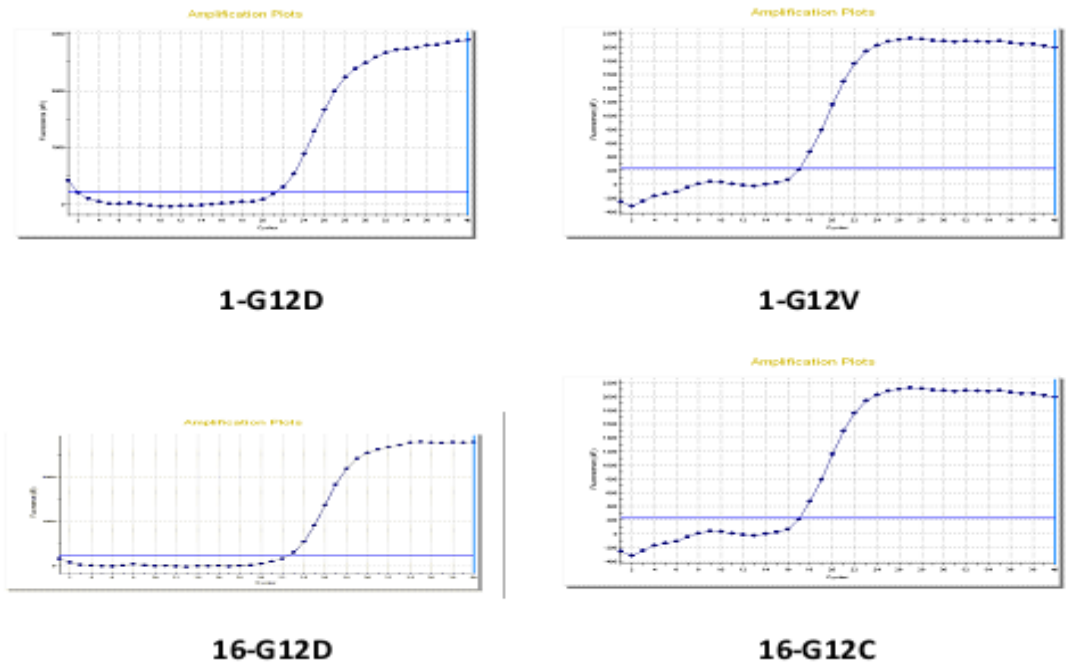
Table 3. The ASR-PCR primers for seven mutations detection

Mutations	Primer (5'-3')	L	%GC	Tm	(1)	(2)	(3)	P (bp)
G12D	F: AAACTTGTGGTAGTTGGAGCGGA	23	47.8	58.6	-0.62	-4.88	-10.26	85
(c.35G>A)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G12V	F: AAACTTGTGGTAGTTGGAGCAGT	23	43.5	56.7	-0.62	-4.88	-10.26	85
(c.35G>T)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G13D	F: GTGGTAGTTGGAGCTGGAGA	20	55.0	56.3	0.13	-6.34	-6.6	79
(c38G>A)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G12C	F: AATATAAACTTGTGGTAGTTGGAGCCT	27	37.0	56.2	-0.62	-4.88	-10.26	90
(c.34G>T)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G12S	F: AATATAAACTTGTGGTAGTTGGAGCGA	27	37.0	56.2	-0.62	-4.88	-10.26	90
(c34G>A)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G12A	F: AACTTGTGGTAGTTGGAGCTTC	22	45.5	55.0	0.5	-6.34	-10.26	84
(c.35G>C)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
G12R	F: AATATAAACTTGTGGTAGTTGGAGCTC	27	37.0	.0	-0.62	-9.49	-10.26	90

Mutations	Primer (5'-3')	L	%GC	Tm	(1)	(2)	(3)	P (bp)
(c34G>C)	R: CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91		
Reverse	CATATTCGTCCACAAAATGATTCTG	25	36.0	52.6	0.79	-3.91	-3.61	
Reference	GACTGAATATAAACTTGTGGTAGTTGGA	28	35.7	54.8	-0.62	-4.88	-10.26	
Probes	CTGTATCGTCAAGGCACT	18	50.0	51.7	0.87	-3.61		

Experimentally, we found out that mutation occurs on codon 12, 13: G12D (sample 1, 16, 37), G12V (sample 1) and G12C (sample 16), particularly in sample 1 existed two mutations GD12V and G12V, sample 16 existed two mutations G12D and G12C (Figure 4)

Figure 4. The positive results of ASR-PCR on sample 1 and 16



According to Lang *et al*. (2011), the ASR-PCR allowed to detect the mutation within less than 1% DNA mutation. Moreover, they also reported that among negative samples were surveyed by PCR-sequencing, there were two positive samples detected by ASR PCR using the primers/probe used in this study.

4. Conclusion

To sum up, we concluded that: (1) By synthesis of literature reviews: prominent mutations on *K-ras* gene were G12D, G12V, G13D, G12C, G12S, G12A, G12R; on *EGFR* gene were deletion E746-A750 on exon 19, point mutation L858R on exon 21; mutation types did not exclude each other and there was no significant differences between racial factors; (2) successfully designing and evaluating PCR-sequencing's primers; primers/probes for ASR-PCR to detect prominent mutations on exon 1 of *K-ras* gene; (3) successfully established DNA extraction protocol from paraffin embedded tissue. Experimentally, PCR-sequencing and ASR-PCR were carried out on 19 biopsy samples from colorectal cancer to successfully record some mutation.

In further study, we would apply this protocol which established on the larger size of sample.

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