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Kras, Braf, PIK3CA and EGFR Gene Mutations are Associated with Lymph Node Metastasis and Right Sided Colon Carcinoma

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Abstract

Mitogen activated proteinase kinase (MAPK) is a key regulator of cellular growth and survival. Mutations in the MAPK pathway genes such as EGFR, KRAS, BRAF and PIK3CA lead to cellular disequilibrium that result into overgrowth and different type of carcinoma including colorectal carcinoma (CRC). Different techniques have been used to determine mutations in the above mentioned genes, and frequency of mutations can vary in different population. The aim of our study was to evaluate the frequency of mutations in the hotspot regions of EGFR, KRAS, BRAF and PIK3CA genes in Swedish colon carcinoma patients by pyrosequencing, and to correlate with different clinicopathological parameters and patient survival. We screened 124 colon cancer patients by using pyrosequencing. We detected Kras, Braf and PIK3CA mutations in 24%, 18.5% and 5.6% of the patients respectively while no mutation was observed for EGFR in our cohort. Kras mutations significantly correlated with lymph node metastasis and advanced UICC stages and poor patient survival (HR; 2.26, 95% CI; 1.273-4.13, log rank P, 0.006). Non-significant correlations were observed between Braf and all parameters including patient survival except with right sided colon cancer. PIK3CA mutations were associated with lymph node metastasis, distant metastasis and higher UICC stages. Combined mutations in Kras, Braf and PIK3CA were significantly associated with lymph node metastasis and colon cancer located on the right flexure. PIK3CA and Kras co-mutations were observed in 4 patients and were significantly associated with lymph node metastasis, distant metastasis and advanced UICC stages.

Keywords: Kras; Braf; PIK3CA; Mutations; Clinicopathological parameters; Patient survival

Introduction

Colorectal cancer is the results of complex interactions between epigenetic, genetic and environmental factors [1]. These factors cause changes in the fine-tuned pathways of normal cell growth and proliferation. Genetic factors are one of the most important factors during which alterations in the genes that are involved in the coding of proteins result in the failure of normal gene functions [2] Like other cancers, development of CRC occurs through genetic deviations in multistep processes that lead to inactivation of tumor suppressor genes and activation of proto-oncogenes by mutation [3].

Proliferating signals from epidermal growth factor receptor (EGFR) result in cellular growth and survival. RAS-RAF-MAPK and RAS-PIK3-PTEN-AKT are two most important alternative pathways that are involved in transferring EGFR mediated cell membrane signals [4,5] Therefore, altered expression, and or mutations in any of the genes encoding for proteins elaborated in proliferation signalling can invoke disequilibrium in cellular growth that may result into various forms of cancer including CRC [6,7].

EGFR, the very first protein in signalling pathway that encounters directly with the ligand, has been reported to be overexpressed in more than 80% of colorectal tumours and a significant association has been established with T3 stage of TNM [7]. However, mutations in this gene have also been reported in CRC [8,9]. Therefore, mutations or overexpression may lead to the activation of EGFR downstream regulated pathways [10].

Until recently, oncogenic mutations in Kras were considered to confer anti-EGFR resistance in metastatic colorectal cancer, and now it has become mandatory to screen CRC patients for Kras mutations before anti-EGFR therapy [11,12]. However, comparatively 30-40% of the nonresponsive patients for such therapies are due to Kras mutations and the rest of the patients with wild type Kras gene are still not responding to the treatment [11,13]. Later on, it has been observed that oncogenic mutation in some other genes of downstream signalling pathways, such as Braf and PIK3CA are alternative proteins that most likely are considered to be responsible for resistance against monoclonal anti-EGFR therapy in metastatic colorectal patients [13-16].

Many studies have been investigating mutations in various genes that are involved in cellular signalling from EGFR to downstream [14,17,18]. However, most of the studies have considered simultaneous screening of Kras, Braf and PIK3CA, three genes in the same patients. Furthermore, different techniques have been used to determine mutation analysis by various earlier investigations and each technique has limitations and advantages [17-19]. Therefore, the aim of the present study was to examine mutations in colon cancer patient samples, in the hotspot regions of EGFR, Kras, Braf and PIK3CA genes by using pyrosequencing, and correlating these mutations with different clinical and pathological parameters individually and in combination, as mutations in these genes govern similar characteristics to the cancerous cells in CRC. Different hotspot regions were studied, codons 712, 711, 710, 707, 795 and 796 in EGFR, 12 and 13 in Kras, 464, 466, 469, 600 and 601 in Braf and in PIK3CA codons 542, 546, 545 and 1047.

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Materials and Methods

Selection of patient's samples

A total of 124 formalin fixed and paraffin embedded (FFPE) samples were selected from patients diagnosed with colon carcinoma from 2002-2009 at Örebro university hospital, Örebro, Sweden. Rectal carcinomas samples were excluded in this study since these patients often are treated with local radiation therapy prior to surgery that can cause changes in the DNA. Samples were randomly collected from both men and women with an average age of 79 (range 40-106 years). Samples were obtained from Orebro University Hospital, Örebro, Sweden. This study was approved from the ethical committee in Uppsala, Uppsala, Sweden.

DNA extraction

In the FFPE tumour blocks, tumour cells rich areas were outlined by an experienced morphologist (V.H-S) with the proportion of tumour cells >70% in the marked area. For genomic DNA extraction, 2 punches á 2 mm diameter from tumour cells rich delineated area of the FFPE tissue sample blocks were taken, and Nucleospin® Nucleic acid and Protein Purification kit (Macherey-Nagel, Germany) was used according to instructions provided by the manufacturer. To determine concentrations and quality of DNA, NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used and extracted DNA samples were stored at -20°C.

Primer designing

Primers for Kras codon 12 and 13 has already been used in earlier study [20]. Primers for EGFR, Braf and PIK3CA were designed by using Pyro Mark Assay Design 2.0 software (Qiagen sample and Assay technology, Hilden, Germany). Information on sequences of the primers (forward, reverse and sequencing primers), their annealing temperatures, size of amplified PCR products, sequence to analyse and codon analysed in the pyrosequencing run are given in Table 1.

PCR and pyrosequencing

For PCR, a master mix of 50 µl was used containing, KAPA2G buffer M (1X), reverse and forward primer (0.25 µM) (Biomers.net, GmbH, Germany), $MgCl_2$ (1 mM), deoxyribonucleotide triphosphate (dNTPs, 200 µM), KAPA2G Fast Hot Start polymerase (1U) (KAPA Biosystem, Boston Massachusetts, USA) and genomic DNA (90-100 ng). PCR reactions were conducted in a thermal cycler 2720 Gene Amp® (Applied Biosystems, Foster city, USA) with the initial denaturation at 95°C for 10 minutes, followed by 49 PCR cycles with denaturation at 94°C for 45 seconds, annealing temperature (according to optimized annealing temperature of each primer set, Table 1) for 30 seconds, and extension at 72°C for 30 seconds. Finally an extension was completed at 72°C for 7 minutes.

Mutations were detected by using PyroMark Q 96 ID pyrosequencer (Qiagen.Biotage AB, Uppsala, Sweden) according to the procedure given by the manufacturer. In summary, 25 µl PCR products were mixed with master mix solution of Streptavidin Sepharose® Beads (GE Healthcare Bio-Science AB, Uppsala, Sweden). Biotinylated strands conjugated with Sepharose beads were washed and transformed to single strands by passing through 70% ethanol, denaturing and washing buffer, and finally were released in the solution of sequencing primer. Mutations were analyzed as SNPs by PyroMark ID 1.0 software (Biotage, Sweden).

Statistics

Quantitative variables were analysed by calculating their means and standard deviation, while of qualitative variables by percentages and frequencies. Statistical associations were determined by Spearman *Chi-square* and Fisher Exact test accordingly. Kaplan Meier log rank test was used to construct survival curves and for survival analysis. Univariate cox regression analyses with confidence interval (CI) of 95% were applied for calculating Hazard's ratio (HR). A two sided p value

Genes	Primers Sequences	†Sequence to analyse	Target Nucleotides	Size (bp)	TM °C
EGFR					
Codon 712, 711, 710	*F-GCCTCTTACACCCAGTGGAGAA R-CGGAGCCCAGCACTTTGAT S-CCAGCACTTTGATCTTT	TTGRAWWCAG YTTCCTTCAA	2135, 2133-2132, 2128	92	58
Codon 796, 795	*F-TGGGCATCTGCCTCACCT R-TTGCGATCTGCACACACCA S-AGTCCAGGAGGCAGC	YGRAGGGCATGAGCT	2386, 2384	135	59
Kras					
Codon 12, 13	*F-TATAAGGCCTGCTGAAAATGACTG R-TTAGCTGTATCGTCAAGGCACTCT S-GTCAAGGCACTCTTGCCTA	CGCNACNAGCTC CGMCANCAGCTC	37, 34 38, 35	87	58
Braf					
Codon 464, 466, 469	*F-ACCATGCCACTTTCCCTTGTA R-GAGATTCCTGATGGGCAGATTAC S-ATTACAGTGGGACAAAGA	ATTGDATCTGNATCATTTGNAAC	1391, 1397, 1406	84	59
Codon 600, 601	F-TAGGTGATTTTGGTCTAGCTACA *R-GTGGAAAAATAGCCTCAATTCTTA S-TGATTTTGGTCTAGCTACA	GWGRAMTCTCGATG	1799, 1801, 1803	111	58
PIK3CA					
Codon 546, 545, 542	*F-GAACAGCTCAAAGCAATTTCTACA R-CATGCTGAGATCAGCCAAATT S-CCATAGAAAATCTTTCTCCT	KCTYAGTGAT TTYAGAGAGA	1636, 1633, 1624	159	58
Codon 1047	*F-TGAGCAAGAGGCTTTGGAGTAT R-CCTGCTGAGAGTTATTAACAGTGC S-GTTGTCCAGCCACCA	TGAWGTGCAT CATTCATTTG	3140	182	57

†Red highlighted characters are international union of pure and applied chemistry (IUPAC) codes; *Biotinylated primer; F Forward Primer; R Reverse primer; S Sequencing Primer; bp Base pairs. Red letter denotes targeted Nucleotides

Table 1: PCR and pyrosequencing primers (forward, reverse and sequencing primers) along with their amplicon sizes and annealing temperatures.

of p<0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 18 software (SPSS, Inc, Chicago, IL, USA).

Results

In this study 124 FFPE colon cancer samples were used. Samples were selected randomly and consisted of 63 men and 61 women with a mean age of 79 and standard deviation of 12.5 years. Different clinical and pathological parameters of the selected patients are provided in the Table 2.

Mutation analysis

Pyrograms of wild type and different mutations in various codons of Kras, Braf and PIK3CA are shown in Figures 1-5. Summary on type of mutations in various codons of the studied genes are presented in Table 3. For EGFR we analysed mutations in codon 710, 711, and 712 and 795 and 796 but, no mutation was observed in the 124 selected tumor samples.

Kras mutations were detected in 30 patient samples, of which codon 12 and 13 harbored 24 (80%) and 6 (20%) mutations respectively (Table 3). None of the patients had a mutation in both codons. The most occurring type of mutation was glycine to serine (43%) followed by glycine to valine (23.3%), only one patient had glycine to alanine mutation. Significant associations were observed between Kras mutations, and lymph node metastasis (p=0.022) and UICC stages (p=0.028) by *Chi square* analysis (Table 2). Similarly, survival analysis showed that patients with Kras mutations died at significantly earlier age than patients having wild type Kras with Hazard's ratio (HR) of 2.26 (CI; 1.27-4.13) and p value of 0.008 (Figure 6A).

We detected Braf mutation in 23 patient samples representing 18.5% of the studied population. Although, Braf was screened for mutations in exon 11 codon 464, 466 and 469 and exon 15 codon 600 and 601, all mutations were observed in codon 600 (Table 3). Braf and Kras mutations were mutually exclusive. Significant association was observed between Braf mutations and right sided colon cancer, in which 20 mutations were detected in the right colon compared to 3 in left colon (p 0 0.004). None of the other clinical and pathological parameters were significantly associated with Braf mutations (Table 2). No association was found between Braf mutations and patient survival (Table 4 and Figure 6B).

A total of 7 mutations were observed in PIK3CA, of which 1 mutation was detected in codon 1047 of exon 20 and 6 in two codons of exon 9 (Table 3). Of 6 mutations in exon 9, one mutation was detected in codon 546 and 5 in codon 545. Out of the 7 patients with PIK3CA mutations, 4 patients harbored concomitant Kras, and one Braf mutation. Mutations in PIK3CA were significantly associated with lymph node metastasis (P=0.045), higher UICC stages (P=0.047) and distant metastasis. None of the other patient clinicopathological parameters showed association with PIK3CA mutations (Table 2). Patients with concomitant Kras and PIK3CA mutations were significantly correlated with lymph node metastasis, distant metastasis and advanced UICC stages (Table 5). Patient with PIK3CA alone and simultaneous Kras mutations did not show significant association with patient survival (Figure 6C and Table 4).

Parameters	N	Kras Mutations		Braf Mutations		PIK3CA Mutation		†Total Mutations	
		N(%)	*P*	N(%)	*P*	N(%)	*P*	N(%)	*P*
Gender			0.75		0.108		1		0.48
Males	63	16(53.3)		8(35)		4(57)		26(47.3)	
Females	61	14(46.7)		15(65)		3(43)		29(52.7)	
Age (Years)			0.86		0.142		0.42		0.46
≥75	43	10 (33.3)		11 (47.8)		1(14)		21(38.2)	
75>	81	20 (66.6)		12(52.2)		6(86)		34(61.8)	
T stages			0.86		0.5		0.351		0.4
T1+2	22	5 (16.7)		3(13)		0(0)		8(14.5)	
T3+4	102	25(83.3)		20(87)		7(100)		47(85.5)	
L node metastasis			0.022*		0.86		0.045		0.061
N0	68	11(36.7)		13(56.5)		1(14)		25(45.5)	
N1+2+3	56	19(63.3)		10(43.4)		6 (86)		30(54.5)	
Distant metastasis			0.59		0.9		0.037		1
M0	118	28(93.3)		22(95.7)		5(71.4)		52(44)	
M1	6	2(6.7)		1(4.3)		2(28.6)		3(50)	
UICC Stages			0.028*		0.84		0.047		0.038*
Stage I+II	67	11(36.7)		12(52.2)		1(1.5)		24(44)	
Stage III+IV	57	19(63.3)		11(47.8)		6(10.5)		31(56)	
Differentiation			0.56		0.94		0.194		0.35
Low	19	4(14.2)		4(20.0)		1(5)		9(18)	
Medium	82	18(64.3)		12(60.0)		3(4)		31(62)	
High	18	6(21.4)		4(20.0)		2(11)		10(20)	
Localization			0.71		0.004*		0.112		0.034*
Right colon	75	19(63.3)		20(87.0)		2(28.6)		39(71)	
Left colon	49	11(36.7)		3(13.0)		5(71.4)		16(29)	

†five patients had mutation in two genes so total do not add to 100%; n, number of mutations in each gene; TNM, tumour node metastasis; M1, metastasis, M0, no metastasis; UICC, Union for international cancer control: *, results are statistically significant

Table 2: Association of different clinicopathological parameters with various mutations.

We further combined the mutation in all three genes to overview general trend of these mutations. We found that mutations were significantly associated with advanced UICC stages and right sided tumours (0.05>P). Trend of increasing association was also observed with lymph node metastasis, but results were non-significant (P=0.061). Association was non-significant with all other clinicopathological parameters (Table 2) and patient survival (Table 4 and Figure 6D).

Discussion

Proteins of the MAPK pathway are well known to play a pivotal role in cellular signalling [5]. Oncogenic mutations in the genes of RAS-RAF-MAPK and PIK3-PTEN-AKT pathways have been reported to involve in initiation and tumour progression of CRC [6,17,18,21]. EGFR, Kras, Braf and PIK3CA are the most reported genes that are either altered due to mutations and or variation in their expression which can effect cellular equilibrium [6,7,17]. Kras mutations have been observed to occur most frequently with reported rates of 20-40 % in colorectal cancers [19,22,23] while mutations in PIK3CA and Braf mutations have been reported comparatively at a lower rate with frequencies of 6-14% and 3-25% respectively [14,17,19]. However, mutations in EGFR have been described as rare event in CRC [8,9]. Mutations in these genes are known to involve not only in initiation and tumour progression, but also confer resistance against anti-epidermal growth factor receptor therapy used to treat colorectal cancer patients [6,14]. Therefore, it is important to determine mutations by using a cost effective, rapid and prudent method to improve therapy management. The aim of this study was to detect mutations in EGFR, Kras, Braf and PIK3CA by using pyrosequencing techniques that has been proven to be highly sensitive technique in mutations detection [24].

In our study, no mutation was observed in EGFR gene in colon cancer patients. Similar results have been reported by Barber TD et al. [25], who found one mutation out of 293 CRC patients. Some other studies have reported mutations in CRC patients at very low rates [8,9]. However, expression of EGFR is known to increase in more than 80% of colon carcinoma [7]. These observations may suggest that EGFR mutations are a rare event, but EGFR overexpression plays a major role in CRC.

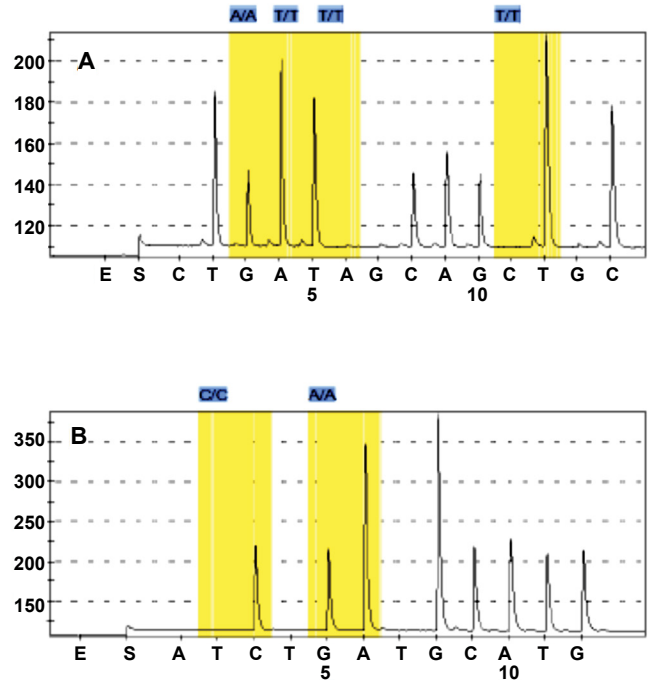

Figure 1: Representative pyrograms of wild type EGFR gene. Figure 1A represent pyrogram of wild type EGFR gene at codon 712, 711 and 710 (highlighted nucleotides; 2135, 2133-2132, 2128) respectively, and 1B wild type EGFR at codon 796 and 795.

We observed Kras mutations in 24% (29/124) of the patients, most of which were detected in codon 12 (Table 3). Frequency of Kras mutations fall within the already reported range (20-40%) in CRC patients [19,22,23]. Possible explanation for such broad range in results can be attributed to different studied populations. Even in the same population [26], different methods have been used, different target codons, and even the proportion of the tumours at various stages (early/advanced) can account for the variety in results. In current study, as mentioned earlier, we excluded rectal samples and also have relatively few mucinous samples; therefore, the choice of samples may possibly contribute to the variation in results. Kras mutations were significantly associated with lymph node metastasis (P=0.022) and UICC stages (P=0.028), but results were non-significant with other clinical and pathological parameters (Table 2). Our results are in agreement with the previous study reports [20,21]. Consistent with the earlier studies, post diagnostic survival in patients harbouring mutated Kras was significantly lower compared to wild type with HR of 2.26 [27,28].

Mutations in Braf were detected in 23 cases constituting 18% of the total population and all mutations were detected in codon 600. Most of the former studies have reported Braf mutations in less than 10% of CRC population [14,23,27], though there is one more recent study report which described this proportion in 25% of CRC cohort [17]. Additionally, we did not find Kras and Braf co-mutations in our samples. Our results are in accordance with previously studies showing that Braf and Kras mutations are mutually exclusive in cancers as reported by Li et al., Davies et al., and Chan et al. [14,29-31]. An association was observed between Braf mutations and tumour localization. Similar results were observed by Liou et al. [23]. Likewise in accordance with the prior studies, mutations in Braf were non-significantly associated with different clinicopathological features such as gender, age, TNM, UICC stages and tumour differentiation [17,23]. Similarly, we also found that Braf mutation were non-significantly associated with overall survival (Table 4). Comparable results have also been reported by other studies [32-34], however most of the former studies have reported

Gene	Nucleotide changes	Amino Acid changes	N (%)
Kras			
Codon 12	c.G34>A	Gly12>Ser12	7(23.3)
	c.G34>T	Gly12>Cys12	4(13.3)
	c.G35>A	Gly12>Asp12	5(16.6)
	c.G35>T	Gly12>Val12	7(23.3)
	c.G35>C	Gly12>Ala12	1(3.3)
Codon 13	c.G37>A	Gly13>Ser13	6(20.0)
Braf			
Codon 600	c.C1799>A	Val600>Glu600	
PIK3 CA	c.G1633>A	Glu545>545Lys	1 (14.3)
	c.C1636>A	Glu546>546Lys	5 (71.4)
	c.A3140>G>G	His1047>Arg1047	1 (14.3)
N: number of mutations in each gene; %: percentage of mutations within each gene			

Table 3: Summary of observed mutations in Kras, Braf and PIK3CA, substituted nucleotides and resulting amino acids and frequency of mutation at each position.

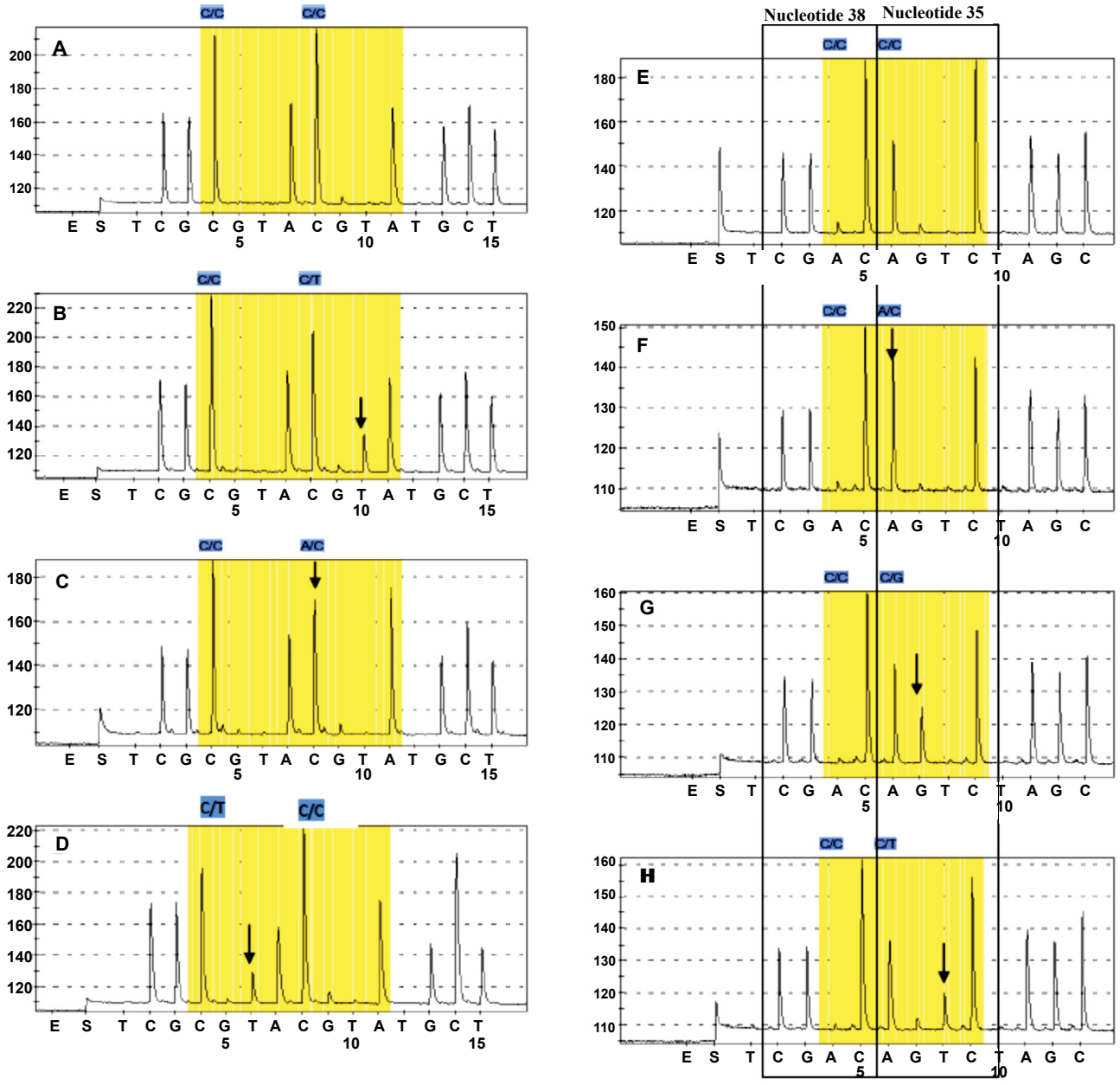

Figure 2: Representative pyrograms of wild type and mutated Kras gene at different nucleotide positions of codon 12 and 13. Figure 2A and 2E represent wild type Kras at nucleotide positions of 37, 34 and 38, 35 respectively. As sequencing primers was reverse therefore coding sequence are complimentary to CGCCACCAGCTC i.e. GAGCTGGTGGG. Pyrograms B, C and D show mutations (pointed by arrows) at codon 12 (GGT>AGT, TGT) and codon 13 (GGT>GAT), while pyrograms F, G and H, at codon 12 (GGT>GTT, GCT, GAT).

that patients with Braf mutations were significantly associated with overall bad survival [14,23,27]. This discrepancy could be attributed to a variation between studied populations, stages at diagnosis and differences in therapy used to treat CRC. Deviation in our results could also be due to relatively higher frequencies of Braf mutations and lower death rate (36% dead after 3 years).

In this study, PIK3CA was found mutated in 7 (5.6%) patients, mutation rate was consistent with earlier studies [19,28] (Table 3). PIK3CA mutations were significantly associated with lymph node metastasis, UICC stages and metastasis, however non-significant associations were observed with other clinical features (Table 2). Such results have also been reported by a previous study [29]. In agreement with former studies, concomitant Kras mutations were frequent in patient samples, and the results were marginally significant (P=0.058). It was interesting to note that co-existing Kras and PIK3CA mutations were significantly associated with lymph node metastasis, distant metastasis and advanced UICC stages (Table 5). Li et al. also found that co-mutations are associated with advanced Duke Stages [29]. From this information we infer that Kras and PIK3CA mutation might have synergistic effects in colon cancer patients. However, from these results, one cannot ruled

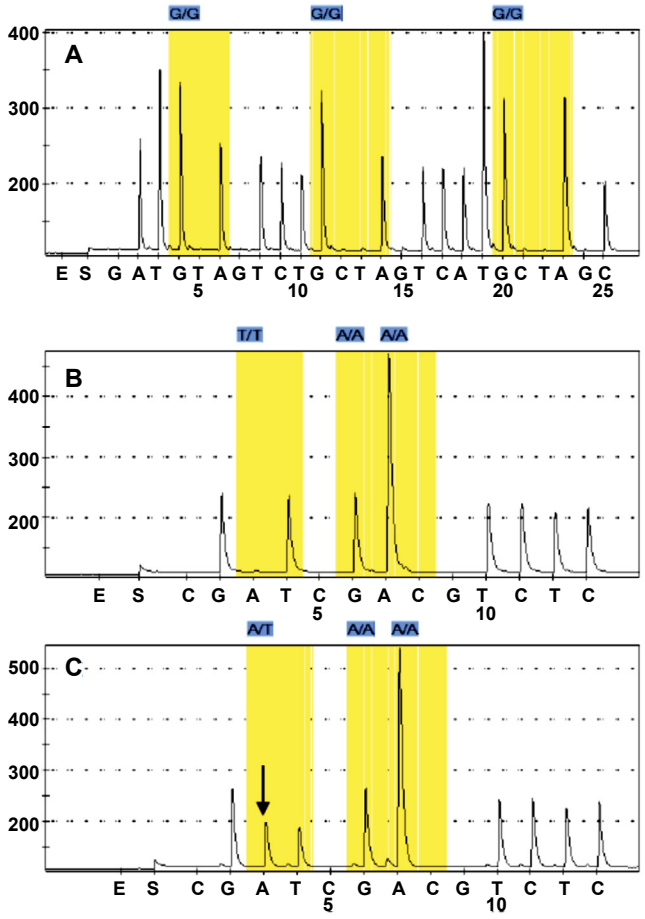

Figure 3: Representative pyrograms of Braf gene. Pyrogram 3A shows wild type Braf at codon 464, 466 and 469 (highlighted nucleotides 1391, 1397, and 1406 respectively). Pyrograms 3B represent wild type Braf at codon 600 and 601, while 3C mutated Braf (pointed by arrow) at codon 600 (GTG>GAG) and wild type at 601 (highlighted nucleotides; 1799, 1801, 1803 respectively).

Genes	†HR	CI (95%)	*P*†† *value*
Kras	2.26	1.273-4.13	*0.006
Braf	0.5	0.2-1.28	0.143
PIK3CA	1.08	0.33-3.49	0.89
PIK3CA-Kras Bi-Mutation	1.14	0.277	0.85
All mutations	1.43	0.797-2.566	0.225

†, Hazard's ration (HR) calculated by Univariate cox regression analysis; ††, Log Rank P value *, statistically significant; CI, confidence interval

Table 4: Survival analysis for patients carrying mutations in different genes.

out the possibility that this combined effect of PIK3CA-Kras co-mutations, may be largely due to the presence of Kras mutations in these patients. In survival analysis, PIK3CA alone, and coexisting with Kras mutations were non-significantly associated with overall patient survival (Table 4).

Combined analysis of Kras, Braf and PIK3CA genes mutations were significantly correlated with advanced UICC stages and right sided colon carcinoma (0.05<P). There was also an increasing tendency for lymph node metastasis (Table 2). These results suggest that these mutations contribute to tumour progression. Soeda et al. showed that the combination of these three genes helped to select the patients who

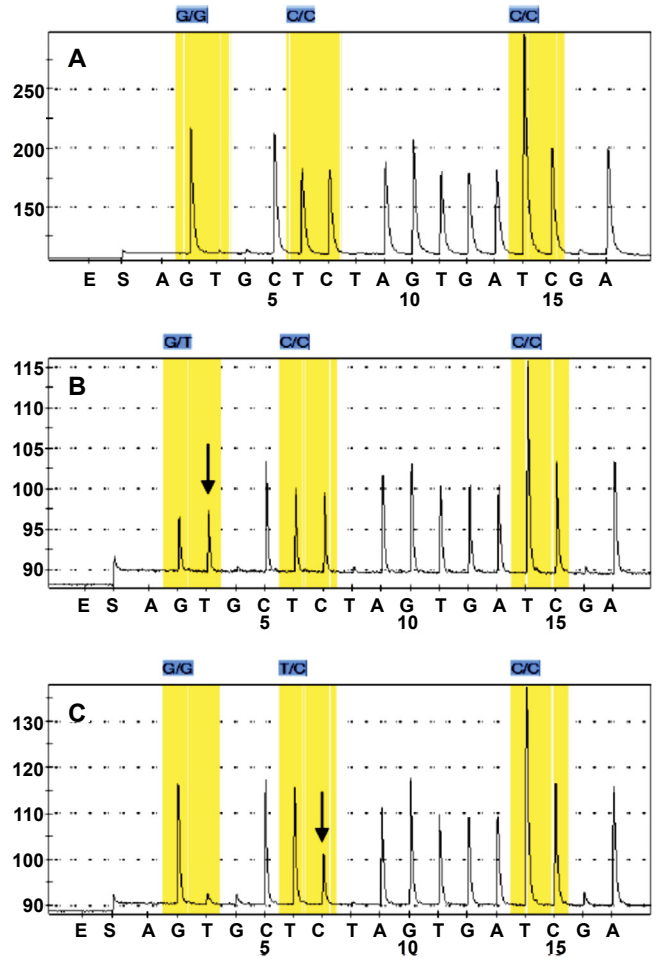

Figure 4: Representative pyrograms of gene PIK3CA codon 546, 545 and 542 (highlighted nucleotides; 1636, 1633, 1624) respectively. As pyrosequencing was performed on complimentary GGCTCAGTGAT TTCAGAGAGA strand, therefore sequencing strand is TCTCTCTGAAATCACTGAGCC. Pyrograms 4A wild type at all three positions, 4B mutated for codon 546 (CAG>AAG) but wild type 545 and 542, and 4C mutated for codon 545(GAG>AAG) and wild type for other two codons.

Characteristics	Kras and PIK3CA Mutations		†P Value
	WT N (%)	Bi-Mut N (%)	
Tumour penetration			1.00
T1+2	22 (18)	0	
T3+4	98(82)	4(100)	
L. Node metastasis			*0.039
N0	68 (57)	0	
N1+2+3	52 (43)	4 (100)	
Distant metastasis			*0.011
Mx	116 (96.7)	2 (50)	
M1	6 (3.3)	2 (50)	
UICC stages			*0.042
I+II	67 (56)	0	
II+IV	53 (42)	4 (100)	

TNM, tumour node metastasis; UICC, Union for international cancer control; †P value calculated by Fischer's exact test; *significant results WT, Wild type for both genes; Bi-Mut, Bi-mutation.

Table 5: Association of PIK3CA and Kras bi-mutation with TNM and UICC stages.

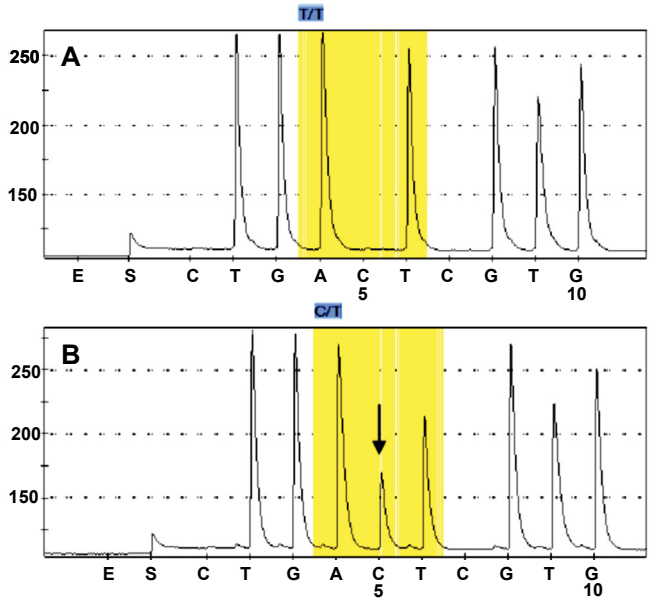

Figure 5: A and B represent wild type and mutated PIK3CA at codon 1047 (CAT>CGT) respectively. Pyrosequencing was performed on complimentary sequence TGATGTG, and sequencing sequence is CACATCA.

most probably are going to benefit from Cetuximab treatment [28]. Regarding survival, results were non-significant between combined mutation and overall patient survival (Table 4).

The strength of our study is that we screened all colon carcinoma patients for EGFR, Kras, Braf and PIK3CA gene mutations. The limitations of our study are that we do not have information of patient therapy that can be useful to further elaborate our findings.

Conclusion

We found that Kras mutations were significantly associated with lymph node metastasis, UICC cancer stages and poor overall survival, whereas significant association was observed between Braf mutation and right colon carcinoma. Mutations in PIK3CA showed significant association with lymph node metastasis, distant metastasis and UICC stages. Concomitant PIK3CA and Kras mutations were frequent and were significantly associated with lymph node metastasis, distant metastasis and advanced UICC stages. Further, mutations in KRAS, BRAF, PIK3CA in combined analysis showed correlation with higher UICC stages and right sided colon cancer, but no correlation with overall patient survival were demonstrated.

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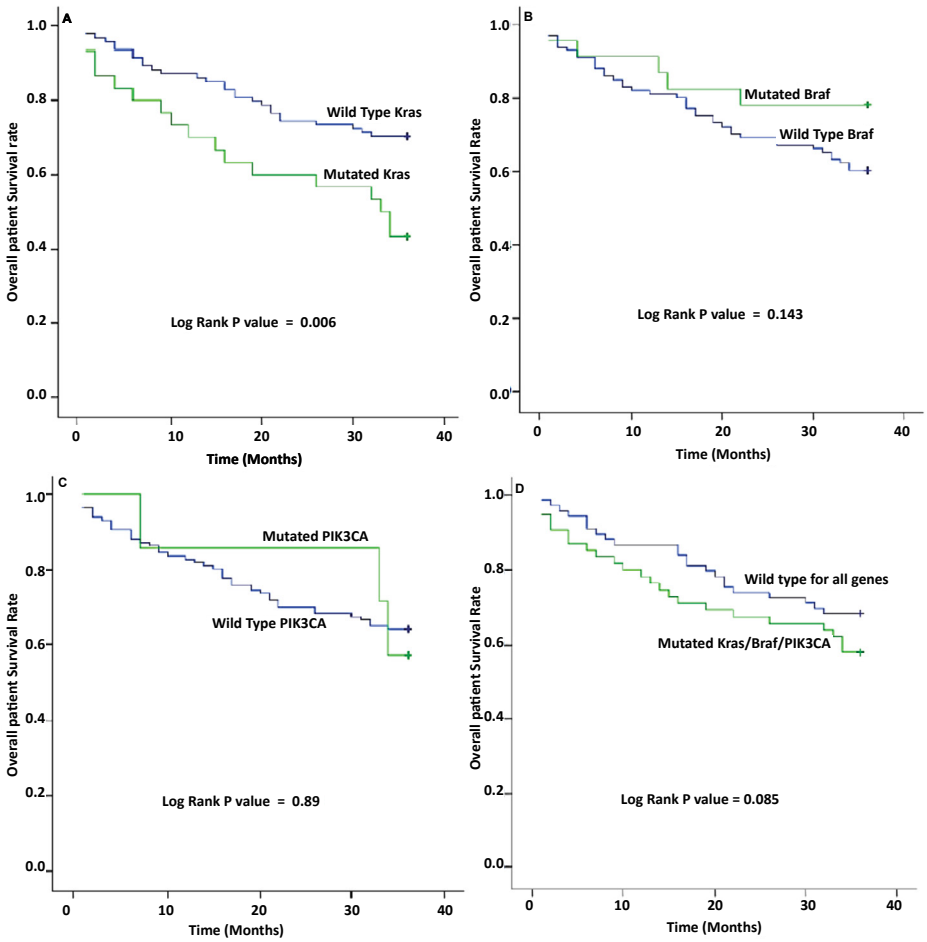

Figure 6: Log rank graphs of survival between mutated and wild type genes. Figure 6A, shows survival between wild type and mutated Kras patients, 6B between, wild type and mutated Braf, 6C between wild type and mutated PIK3CA and 6D between patients with mutation in any gene and wild type for all screened genes.

References

1. Lynch HT, de la Chapelle A (2003) Hereditary colorectal cancer. N Engl J Med 348: 919-932.
2. Berg M, Danielsen SA, Ahlquist T, Merok MA, Ågesen TH, et al. (2010) DNA sequence profiles of the colorectal cancer critical gene set KRAS-BRAF-PIK3CA-PTEN-TP53 related to age at disease onset. PLoS One 5: e13978.
3. Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87: 159-170.
4. Baselga J (2001) The EGFR as a target for anticancer therapy--focus on cetuximab. Eur J Cancer 37 Suppl 4: S16-22.
5. Zhang W, Liu HT (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 12: 9-18.
6. Barault L, Veyrie N, Jooste V, Lecorre D, Chapusot C, et al. (2008) Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. Int J Cancer 122: 2255-2259.
7. Spano JP, Fagard R, Soria JC, Rixe O, Khayat D, et al. (2005) Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. Ann Oncol 16: 189-194.
8. Metzger B, Chambeau L, Begon DY, Faber C, Kayser J, et al. (2011) The human epidermal growth factor receptor (EGFR) gene in European patients with advanced colorectal cancer harbors infrequent mutations in its tyrosine kinase domain. BMC Med Genet 12: 144.
9. Tsuchihashi Z, Khambata-Ford S, Hanna N, Jänne PA (2005) Responsiveness to cetuximab without mutations in EGFR. N Engl J Med 353: 208-209.
10. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, 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.
11. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, et al. (2009) American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 27: 2091-2096.
12. van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, et al. (2008) KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch 453: 417-431.
13. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, et al. (2008) Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 26: 5705-5712.
14. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, et al. (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 11: 753-762.
15. Rizzo S, Bronte G, Fanale D, Corsini L, Silvestris N, et al. (2010) Prognostic vs predictive molecular biomarkers in colorectal cancer: is KRAS and BRAF wild type status required for anti-EGFR therapy?. Cancer Treat Rev 36 Suppl 3: S56-S61.
16. Sartore-Bianchi A, Di Nicolantonio F, Nichelatti M, Molinari F, De Dosso S, et al. (2009) Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. PLoS One 4: e7287.
17. Mao C, Zhou J, Yang Z, Huang Y, Wu X, et al. (2012) KRAS, BRAF and PIK3CA mutations and the loss of PTEN expression in Chinese patients with colorectal cancer. PLoS One 7: e36653.
18. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelin R, et al. (2008) BRAF, KRAS and PIK3CA mutations in colorectal serrated polyps and cancer: primary or secondary genetic events in colorectal carcinogenesis? BMC Cancer 8: 255.
19. Kwon MJ, Lee SE, Kang SY, Choi YL (2011) Frequency of KRAS, BRAF, and PIK3CA mutations in advanced colorectal cancers: Comparison of peptide nucleic acid-mediated PCR clamping and direct sequencing in formalin-fixed, paraffin-embedded tissue. Pathol Res Pract 207: 762-768.
20. Mannan A, Hahn-Strömberg V (2012) K-ras mutations are correlated to lymph node metastasis and tumor stage, but not to the growth pattern of colon carcinoma. APMIS 120: 459-468.
21. Oliveira C, Velho S, Moutinho C, Ferreira A, Preto A, et al. (2007) KRAS and BRAF oncogenic mutations in MSS colorectal carcinoma progression. Oncogene 26: 158-163.
22. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, et al. (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359: 1757-1765.
23. Liou JM, Wu MS, Shun CT, Chiu HM, Chen MJ, et al. (2011) Mutations in BRAF correlate with poor survival of colorectal cancers in Chinese population. Int J Colorectal Dis 26: 1387-1395.
24. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, et al. (2005) Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 7: 413-421.
25. Barber TD, Vogelstein B, Kinzler KW, Velculescu VE (2004) Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 351: 2883.
26. Palomba G, Colombino M, Contu A, Massidda B, Baldino G, et al. (2012) Prevalence of KRAS, BRAF, and PIK3CA somatic mutations in patients with colorectal carcinoma may vary in the same population: clues from Sardinia. J Transl Med 10: 178.
27. Richman SD, Seymour MT, Chambers P, Elliott F, Daly CL, et al. (2009) KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. J Clin Oncol 27: 5931-5937.
28. Soeda H, Shimodaira H, Watanabe M, Suzuki T, Gamoh M, et al. (2012) Clinical usefulness of KRAS, BRAF, and PIK3CA mutations as predictive markers of cetuximab efficacy in irinotecan- and oxaliplatin-refractory Japanese patients with metastatic colorectal cancer. Int J Clin Oncol 18: 670-677
29. Li HT, Lu YY, An YX, Wang X, Zhao QC (2011) KRAS, BRAF and PIK3CA mutations in human colorectal cancer: relationship with metastatic colorectal cancer. Oncol Rep 25: 1691-1697.
30. Chan TL, Zhao W, Leung SY, Yuen ST; Cancer Genome Project (2003) BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res 63: 4878-4881.
31. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, et al. (2002) Mutations of the BRAF gene in human cancer. Nature 417: 949-954.
32. French AJ, Sargent DJ, Burgart LJ, Foster NR, Kabat BF, et al. (2008) Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. Clin Cancer Res 14: 3408-3415.
33. Yoshitake N, Fujii S, Mukawa K, Tominaga K, Fukui H, et al. (2007) Mutational analysis of the BRAF gene in colorectal mucinous carcinoma in association with histological configuration. Oncol Rep 17: 9-15.
34. Maestro ML, Vidaurreta M, Sanz-Casla MT, Rafael S, Veganzones S, et al. (2007) Role of the BRAF mutations in the microsatellite instability genetic pathway in sporadic colorectal cancer. Ann Surg Oncol 14: 1229-1236.