ISSN: 0975-833X

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

Vol.6, Issue 09, September - 2014

Impact Factor: SJIF : 3.845
Indexing: Thomson Reuters: ENDNOTE

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 6, Issue, 09, pp.8952--8957, September, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

DETECTION OF *KRAS* MUTATION IN CIRCULATING FREE DNA (cfDNA)

***Wafaa Sabri Mahood, Muhaamed Ibraheem Nadir and Khalid Tobal**

College of Education, Ibin Al-Haitham, University of Baghdad, Iraq

ARTICLE INFO

Article History:
Received 02nd June, 2014
Received in revised form
31st July, 2014
Accepted 19th August, 2014
Published online 30th September, 2014

Key words:
Monitoring,
Mutations,
cfDNA,
KRAS.

ABSTRACT

There are multiple potential uses for cfDNA in cancer diagnosis and prognosis for example, cfDNA can be used to detect somatic alterations when biopsies are not available. It may also represent a valuable source of tumor DNA when the exact position of a suspected primary lesion is not clearly defined. Aside from these clinical applications, cfDNA may also represent a very important source of biomarkers in population-based studies. The fact that cfDNA can be obtained without invasive or painful procedures makes it particularly. We aimed to detected if there is any mutations in KRAS as genetic marker could be find in plasma samples from Iraqi patients as early diagnosis and compared the results with match tumor. Material and methods: Plasma samples for this study were collected in addition tumor tissue was collected via biopsy from 18 patients at early diagnosis of colorectal cancer. DNA was extracted from frozen tumor tissue and cfDNA was extracted from the plasma samples. KRAS mutations were detected by High Resolution Melting (HRM). Results: There is (33%) of patients tumour tissue with *KRAS* mutation in codon 12, *KRAS* mutation in sequencing analysis was successful only in (7%) of the plasma-extracted cfDNA sample which didn't mutate in the matched tumor. Conclusion: We found it is unlikely that circulating mutant DNA measurements be used to reliably monitor tumor diagnostic and monitoring potential of detecting of *KRAS* oncogene in plasma from Iraqi patients as early diagnosis based on the fact that we were unable to detect such DNA in plasma and match tumor tissue.

Copyright © 2014 Wafaa Sabri Mahood et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Tumour derived mutant DNA has been detected in various body fluids. Mutations in various genes, including KRAS2, p53, and CD44 among others, have been detected in stool samples of patients with CRC (Villa *et al.,* 1996; Dutta and Nair 1998; Ahlquist *et al.,* 2000). The presence of abnormally high levels of free circulating DNA (cfDNA) in the plasma/serum of cancer patients was demonstrated in 1977. (Leon *et al.,* 1977), The importance of cfDNA was recognized in 1994 as a result of the detection of mutated RAS gene fragments in the blood of cancer patients (Vasioukhin *et al.,* 1994; Sorenson *et al.,* 1994). Mutations in cfDNA have been characterized in a large variety of cancer types and sites, including, for example, colorectal, pancreas, lung, bladder, head and neck and liver cancers Various types of DNA alterations have been reported in cfDNA, including point mutations, DNA hypermethylations, microsatellite instabilities (MI) and losses of heterozygosity (LOH). In many instances, these alterations were identical to the ones found in the primary tumor tissue of the patient, supporting the tumoral origin of altered cfDNA. Occurrence of alterations in cfDNA, as well as increase in the overall level of cfDNA, is not restricted to any particular tumor site, type or grade (Gormally *et al.,* 2007).

***Corresponding author: Wafaa Sabri Mahood**
College of Education, Ibin Al-Haitham, A-Aadhamia, University of Baghdad, Iraq.

There are multiple potential uses for cfDNA in cancer diagnosis and prognosis. For example, cfDNA can be used to detect somatic alterations when biopsies are not available. It may also represent a valuable source of tumor DNA when the exact position of a suspected primary lesion is not clearly defined. Moreover, it may provide a useful way to detect early relapse during post treatment follow-up of patients with defined mutations in the primary lesion. Aside from these clinical applications, cfDNA may also represent a very important source of biomarkers in population-based studies. The fact that cfDNA can be obtained without invasive or painful procedures makes it particularly (Northman *et al.,* 1995). Mutant KRAS2 has been detected in DNA extracted from pancreatic fluid in patients with pancreatic carcinoma, from urine specimens in patients with bladder cancer, and from the sputa of patients with lung cancer. Tumour derived circulating mutant DNA is also detectable in the plasma or serum of cancer patients, as indeed is tumour derived RNA. Circulating wild-type DNA is detectable at low levels in normal healthy controls (Ryan *et al.,* 2003).

Recent study provide evidence that detection of *KRAS* mutational status in circulating tumor cells, by gene expression array, has potential for clinical application in selecting metastatic colorectal cancer patients most likely to benefit from cetuximab therapy (Li-ChenYen *et al.,* 2009). *KRAS* analysis in

plasma is a viable alternative to tissue analysis. Quantitative levels of cfDNA and pm *KRAS* are strongly correlated and hold promise of clinical application (Spindler *et al.,* 2012). We aimed to detected if there is any mutations in *KRAS* as genetic marker could be find in plasma samples from Iraqi patients as early diagnosis and compared the results with match tumor.

MATERIALS AND METHODS

Patients

Plasma samples for this study were collected in addition tumor tissue was collected via biopsy from 18 patients at early diagnosis of colorectal cancer who underwent to the Gastroenterology and Hepatology Diseases Center, and Teaching Laboratories In Baghdad, between November, 2011 and May, 2012. The 6 male and 12 female with a median age of 54 years (range, 25–72 years). patients were classified as 4 patients with well differentiated tumor, 14 patients with moderately differentiated tumor according to the Modified Dukes classification of (Astler and Coller 1954) Astler and Coller (1954), as diagnosed by colonoscopy, biopsy and confirmed by histopatholgist examination. Patients treated with radiotherapy or chemotherapy were excluded. Blood samples (5ml) were collected from each Patient in EDTA tube after fasting and the plasma was separated by centrifugation at 3,000 x g for 2.5 minutes at room temperature then plasma was transferred to a clean tube. The plasma and tissue samples were stored at -20 °C until use for the studies. All patients were informed about the aims of this study which includes studies for publication of this report or any accompanied images. and, before the samples was collected, gave a written informed consent. The research was approved by council of Institute of Genetic Engineering and Biotechnology for Post Graduate Studies. University of Baghdad.

DNA extraction and *KRAS* mutation analysis

DNA was extracted from frozen tumor tissue using the QIAamp DNA Mini and Blood Mini kit according to the manufacturer's instructions.

cfDNA was extracted from the plasma samples according to the (Yuan *et al.,* 2012) with modification . The frozen tumour tissues were collected and processed at the laboratory of the National Center for Early Detection of Cancer (Baghdad) while the Plasma cfDNA were extracted at the Laboratory of Molecular Oncology at Guy 's Hospital (London).

The coding sequence and splice junctions of exons 2 and 3 in *KRAS* gene where all pathogenetic mutations occur (Bardelli and Siena 2010) includes the most common mutation sites (codons 12 and 13) using the following primer pairs (forward, 5´TTATAAGGCCTGCTGAAAAT GACTGAA and reverse 5´TCATGAAAATGGTCAGAGAA ACC. *KRAS* mutations were detected by High Resolution Melting (HRM). Briefly, HRM was performed on (4 µl of isolated genomic DNA, PCR buffer 2 µl , $MgCl_2$ (26mm) 1.28 µl, dNTPs 0.35 µl enzyme 0.25 µl,water 10.5 µl,dye 1 µl and primer mix 0.62 µl) using HRM-ABI in a 9700 Thermal cycler (Applied Biosystems, Foster City, CA, USA); all HRM amplified products were cleanup using ChargeSwich®PCR Clean-UP Kit. Then directly sequenced. The sequence was analyzed using the ABI 3730 (Applied Biosystems).

Statistical Analysis

*The Statistical Analysis System- SAS (2004) was used to effect of difference factors in study parameters. (SAS 2010)

RESULTS AND DISCUSSION

Detection of *KRAS* mutation in circulating Free DNA Frozen tumor extracted DNA

We aimed to detected if there is any mutations in genetic marker could be find in plasma samples from patients as early diagnosis and compared the results with match tumor. Plasma samples for *KRAS* mutation analysis were available from 18 patients with their match tumor tissue in early CRC. Three of plasma extracted DNA were failed to detected of *KRAS* mutation. Overall, 15 (100%) of the plasma DNA and frozen tissue samples a were analyzed successfully for *KRAS*1213 by HRM Figure (1).

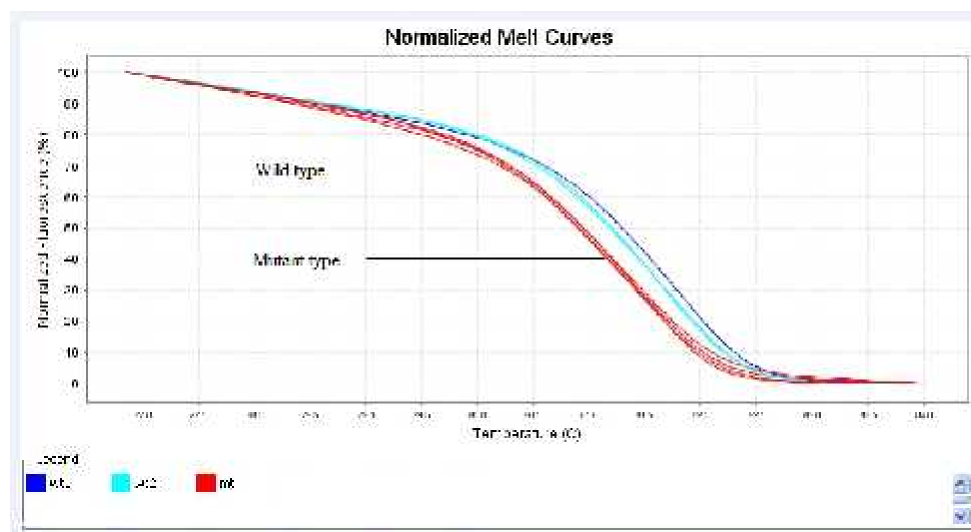

Figure 1. HRM melting profile result of the *KRAS* gene codon 1213 isolated from colorectal tissue. The figure shows the results of Gene Scanning analyses of wild type samples (blue line) and mutant (red line)

Select the amplified products of mutations samples and purified, after that we confirmed these mutations by sequencing analysis. Of the seven mutations in codons12 and 13 of *KRAS* that detect (G12A, G12R, G12D, G12C, G12S, G12V, and G13D; NCBI reference sequence: NM-004985), there is (33%) of patients 5(15) with *KRAS* mutation in codon 12, the results were included 4(26%) transition, GAT(G12D) and 1(7%) transversion, CGT(G12R) in codon 12 Figure (2) Table (1). Analysis is shown GGT>GATand GGT>CGT mutation. These mutations appear in 11(15)73%, 3(15) 20% and 1(15)7% in moderately differentiated, well differentiated and tumor villous respectively Table (1), while other mutation cannot be detected. There is not mutation in codon 13.10 (15) 67% was the percent of wild type with age rate (47.5 years).Regards to the age the mutations were high significant (P<0.01).

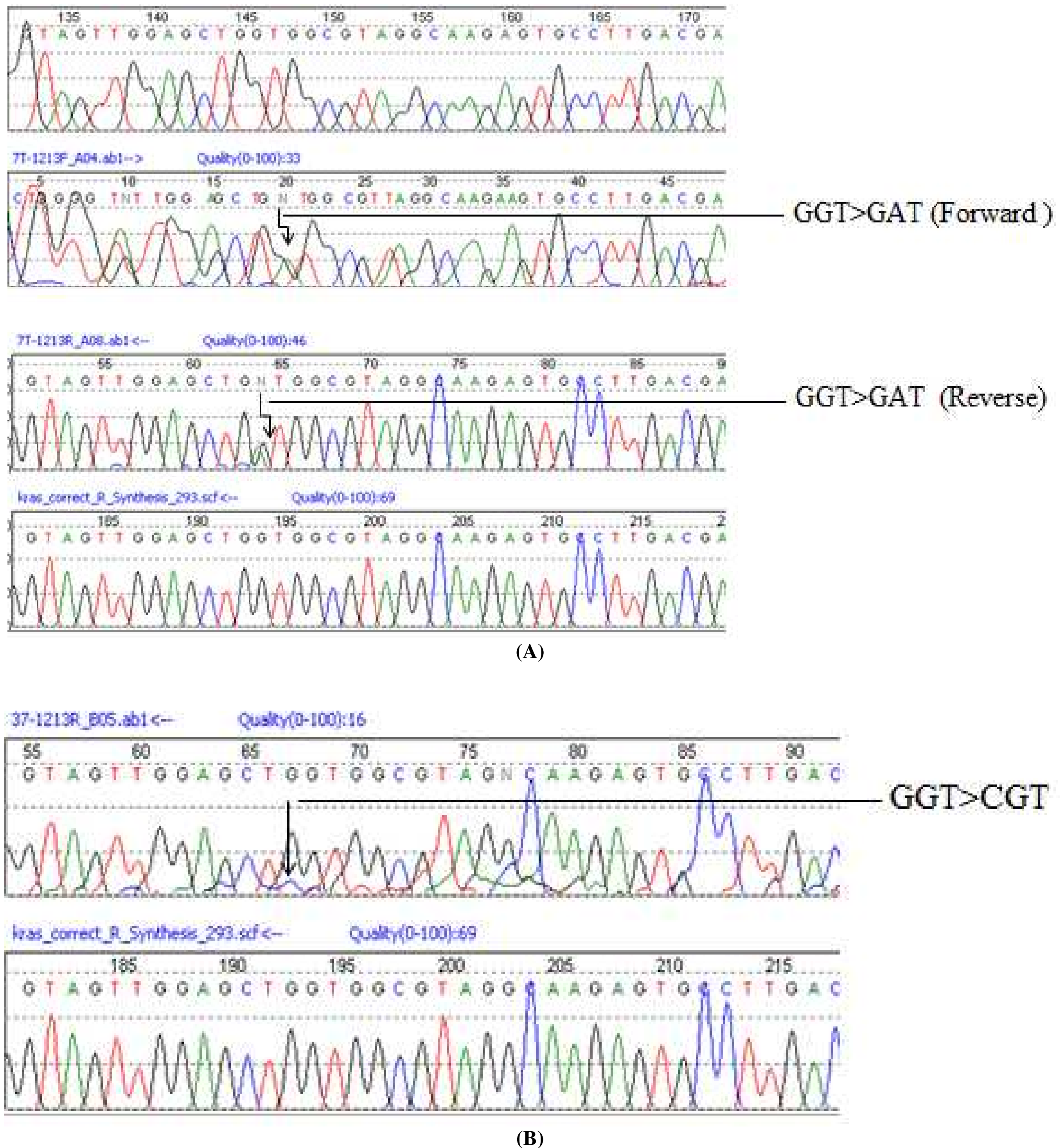

Figure 2. Sequence analyses of *KRAS* gene in DNA isolated from colorectal tissues, the representative sequence analysis is shown for some mutation. (A): Gly12Asp (B): Gly12Val

Table 1. comparison of *KRAS* mutation in tumor DNA and cfDNA in plasma

		R.HRM Plasma		R.HRM Tissue	
Plasma No.	Stage disease	1213	R. of seq.	1213	R. of seq
1cf	Tumor villous	wt		m	GGT>CGT
2cf	M	wt		m	GGT>GAT
3cf	M	wt		wt	
4cf	M	m		wt	
5cf	W	wt		wt	
6cf	M	wt		m	GGT>GAT
7cf	M	m		wt	
8cf	M	m	GGT>GAT	wt	
9cf	M	m		wt	
10cf	M	m		wt	
14cf	W	wt		m	GGT>GAT
15cf	M	m		wt	
16cf	W	wt		wt	
17cf	M	m		m	GGT>GAT
18cf	M	wt		wt	

No- Number, R-Result, Seq-sequence, M- Moderately differentiated, W-Well differentiated, m-mutant, wt-wild

Table 2. Characteristics of colorectal cancer patients and clinicopathology features of tumors with *K RAS* gene mutation status

Character	%	*KRAS* mutation +ve %	*KRAS* mutation - ve %	P- value	2 test
All patients	15(100)				
Age yrs	47.5				
Average age					
>50	13(87)	4(31)	9(69)	0.014	8.904**
< 50	2(13)	1(50)	1(50)	1.00	0.00NS
Gender					
Male	5(33)	3(60)	2(40)	0.0133	6.317 **
Female	10(67)	2(20)	8(80)	0.0048	10.419 **
Site location					
Right	7(47)	3(43)	4(57)	0.0429	3.944 *
Left	3(20)	0	3(100)	0.0016	14.50 **
Rectum	5(33)	2(40)	3(60)	0.0133	6.317 **

* (P<0.05), ** (P<0.01).

In 31% in age over 50 years. Comparison depend on the gender 3(5) 60% also high significant (P<0.01) of *K RAS* mutation were in the male. Against 2 (5) 20% in female, while according to the site it were 3(7) 43% and 2(7) 40% in right and rectum respectively as in table (2) showing low and high significant increase of (P<0.05, P<0.01) respectively of *KRAS* mutation for codon 12, while no *KRAS* mutation in left site of colon.

Utility of cfDNA for *KRAS* mutation analysis

Matched plasma were available from 15 patients with analyzed biopsy tumor samples. We found 7(15) 47% rate of mutation which detected by HRM but failed to detect these mutation except o only one 1(15) 7% of mutation in sequencing analysis, as showed in Table (1). The detection of point mutations in genes, such as TP53 and KRAS in cfDNA requires the use of more sensitive approaches than for the analysis of DNA extracted from tumor tissues. In most cases, direct sequencing has proven to be not sensitive enough, as this method does not allow the reliable detection of less than 25% mutant signal in a background of wild-type DNA (Jenkins *et al.,* 2003).

Plasma-extracted cfDNA versus tumor-extracted DNA

KRAS mutation in sequencing analysis was successful only in 1/15 (7%) of the plasma-extracted cfDNA sample number 8cfDNA which didn't mutate in the matched tumor when tested in HRM and it was transition mutation GGT>GAT(G>D) Table (1). That may due to many reason, first is the mutant DNA represents only a small fraction of total cfDNA (Diehl *et al.,* 2008), second may due to sampling of blood (Kopreski *et al.,* 1997). The third reason, it has been scientifically proven that tumours represent a mixture of different cancer cell clones (which account for the genomic and epigenomic heterogeneity of tumours) and other normal cell types, such as haematopoietic and stromal cells. Thus, during tumour progression and turnover both tumour-derived and wild-type (normal) cfNA can be released into the blood. As such, the proportion of cfNA that originates from tumour cells varies owing to the state and size of the tumour. The amount of cfNA is also influenced by clearance, degradation and other physiological filtering events of the blood and lymphatic circulation. Nucleic acids are cleared from the blood by the liver and kidney and they have a variable half-life in the circulation ranging from 15 minutes to several hours (Fleischhacker and Schmidt 2007). In addition to the mutant DNA fragments present in the circulation of cancer patients are degraded compared with the circulating DNA derived from non neoplastic cells (Diehl *et al*., 2005). The results of studies in the this field of contradictory, Diehl *et al*. (2005) reported it is unlikely that circulating mutant DNA could be used to detect premalignant tumors, based on the fact that they were unable to detect such DNA even in very large adenomas. While other study published that *KRAS* analysis in plasma is a viable alternative to tissue analysis. Quantitative levels of cfDNA and primary metastasis *KRAS* are strongly correlated and hold promise of clinical application (Spindler *et al*., 2012). The cfDNA appears to be a viable tool for the monitoring of the clinical progression of CRC in patients with cfDNA positivity prior to surgery (Levy *et al*., 2012; Lefebure *et al*., 2010).

Previous study have confirmed that cfDNA can be used for the detection of *KRAS* mutations in CRC (Lecomte *et al*., 2010).

Perkins *et al*. (2012) published that multiplex sequencing assay can be utilized to detect somatic mutations from plasma in advanced cancer patients, when safe repeat tumor biopsy is not feasible and genomic analysis of archival tumor is deemed insufficient (Perkins *et al*., 2012). Our results showed that only one sample (17cf) was have compatible results for HRM assay in tissue and match plasma and this mutation was confirmed in sequencing assay in tissue but did not in match plasma, this may explain by that, the tumor occurs frequently at a specific genomic site. A major drawback of cfDNA assays is the low frequency of some of the mutations that occur in tumors. In general, wild-type sequences often interfere with cfDNA mutation assays. This is due to the low level of cfDNA mutations and the dilution effect of DNA fragments and wild-type DNA in circulation (Wang *et al.,* 2010). Conclusion: We found it is unlikely that circulating mutant DNA measurements be used to reliably monitor tumor diagnostic and monitoring potential of detecting of *KRAS* oncogene in plasma from Iraqi patients as early diagnosis based on the fact that we were unable to detect such DNA in plasma and match tumor tissue, the pathophysiological function and clinical implication of their presence warrant further investigation.

REFERENCES

Ahlquist, D., Skoletsky, J., Boynton K, *et al.* 2000. Colorectal cancer screening by detection of altered human DNA in stool: Feasibility of a multitarget assay panel. Gastroenterology. 119:1219–27.

Astler, V.B. and Coller, F.A. 1954. The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann. Sur. 139:846-852.

Bardelli, A. and Siena, S. 2010. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. *J Clin Oncol*. 28:1254–1261.

Diehl, F., Li,M., Dressman, D., He, Y., Shen, D., Szabo, S., Jr, L.D., Goodman, S.N., David, K.A.., Juhl, H., Kinzler, K.W. and Vogelstein, B. 2005. Detection and quantification of mutations in theplasma of patients with colorectal tumors. Acad.Sci.USA102:(45) 16369.

Diehl, F.;Schmidt, K.; Choti,M.A., Romans, K.;Goodman, S.; Li, M.; Thornton, K.; Agrawal, N.; Sokoll, L.; Szabo, S.A.; Kinzler, K.W.; Vogelstein, B. and Diaz, L.A.Jr. 2008. Circulating mutant DNA to assess tumor dynamics. Nat. Med. 14:985–90.

Dutta, S. and Nair, P. 1998. Noninvasive detection of colorectal cancer by molecular tools: Coming of age. Gastroenterology. 114:1333–5.

Fleischhacker, M.and Schmidt, B. 2007. Circulating nucleic acids (CNAs) and cancer-a survey. Biochim. Biophys. Acta 1775, 181–232 (2007).

Gormally, E., Caboux, E., Vineis, P. and Hainaut P. 2007. Review:Circulating free DNA in plasma or serum as biomarker of carcinogenesis: Practical aspects and biological significance. Models Murer.7839.

Jenkins, G.J., Doak, S.H., Griffiths, A.P., Tofazzal, N., Shah, V., Baxter, J.N. and Parry, J.M. 2003. Early p53 mutations in nondysplastic Barrett's tissue detected by the restriction site mutation (RSM) methodology, *Br. J. Cancer*. 88: 1271–1276.

Kopreski ,M.S.; Benko, F.A.; Kwee, C.; Leitzel, K.E.; Eskander ,E.;Lipton, A.andGocke CD. 1997. Detection of mutant K-ras DNA in plasma or serum of patients with colorectal cancer. *Br. J. Cancer.* 1997; 76(10):1293-9.

Lecomte T., Ceze N., Dorval E., Laurent-Puig P. 2010. Circulating free tumor DNA and colorectal cancer. Gastroenterol Clin. Biol. 34:662–81

Lefebure, B., Charbonnier, F., Di Fiore, F., Tuech, J.J., Le Pessot, F., Michot, F., Michel, P. and Frebourg, T. 2010. Prognostic value of circulating mutant DNA in unresectable metastatic colorectal cancer. *Ann Surg*. 251(2):275-80.

Leon, S.A., Shapiro, B., Sklaroff, D.M., Yaros, M.J. 1977. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res*. 37. 646–650

Levy, M., Benesova, L., Lipska, L., Belsanova, B., Minarikova, P., Veprekova, G., Zavoral, M.and Minarik, M. 2012. Utility of cell-free tumour DNA for post-surgical follow-up of colorectal cancer patients. Anticancer Res. 32(5):1621-6.

Li-ChenYen, Yung-SungYeh, Chao-Wen Chen, Hwei-MingWang, Hsiang-LinTsai, Chien-Yu Lu, Yu-Tang Chang, 5 Koung-Shing Chu, Shiu-Ru Lin, andJaw-YuanWang 2009. Detection of KRAS Oncogene in Peripheral Blood as a Predictor of the Response to Cetuximab Plus Chemotherapy in Patients with Metastatic Colorectal Cancer. *Clin. Cancer Res*. 15(13).

Northman, N., Stewart, W.F. and Schulte, P.A. 1995. Incorporating biomarkers into cancer epidemiology: a matrix of biomarker and study design categories, Cancer Epidemiol. *Biomarkers Prev*. 4 (1995) 301–311.

Perkins, G., Yap, T.A., Pope, L., Cassidy, A. M., Dukes, J. P., Riisnaes, R., Massard, C., Philippe A. Cassier, P.A. Miranda, S., Clark, J., Denholm, K.A., Thway, K.., De Castro, D.G., Attard, G., Molife, L. R., Kaye, S.B., Banerji, A. and de Bono J.S. 2012. *Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. PLoS ONE 7(11): e47020. PLoS ONE 7(11): e47020

Ryan, B. M., Lefort, F., McManus, R., Daly, J. Keeling, P. W. N., Weir, D. G. and Kelleher D. 2003. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. Gut. 52(1): 101–108.

SAS. 2010. Statistical Analysis System, User's Guide Statistical. Version 9.1 ed. SAS.Inc. Cary. N.C. USA.

Sorenson, G. D., Pribish, D.M., Valone, F.H., Memoli, V.A., Bzik, D.J. and Yao, S.L. 1994. Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol. Biomarkers Prev*. 3: 67–71.

Spindler, K.G., Pallisgaard, N., Vogelius, I. and Jakobsen, A. 2012. Quantitative Cell-Free DNA, KRAS, and BRAF Mutations in Plasma from Patients with Metastatic Colorectal Cancer during Treatment with Cetuximab and Irinotecan. *Clin. Cancer. Res.*18:1177-1185.

Spindler, K.G., Pallisgaard, N., Vogelius, I. and Jakobsen, A. 2012. Quantitative Cell-Free DNA, KRAS, and BRAF Mutations in Plasma from Patients with Metastatic

Colorectal Cancer during Treatment with Cetuximab and Irinotecan.*Clin. Cancer Res.* 18:117.

Vasioukhin, V., Anker, P. Maurice, P. Lyautey, J. Lederrey, C. and Stroun, M. 1994. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *Br. J. Haematol*. 86:774–779.

Villa, E., Dugani A., Rebecchi, A. *et al.* 1996. Identification of subjects at risk for colorectal carcinoma through a test based on K-ras determination in the stool. Gastroenterology. 110:1346–53.

Wang, S., An, T., Wang, J., Zhao, J., Wang, Z., Zhuo, M., Bai, H., Yang,L., Zhang, Y., Wang, X., Duan, J., Wang, Y., Guo, Q. and Wu, M. 2010. Potential clinical significant of plasma -based KRAS mutation analysis in patients with advanced non-small cell lung cancer. *Clin. cancer Res.* 16:1324-1330.

Yuan, H., Zhu, Z., Lu, Y., Liu, F., Zhang, W., Huang, G., Zhu, G. and Bin Jiang 2012. A Modified Extraction Method of Circulating Free DNA for Epidermal Growth Factor Receptor Mutation Analysis. *Yonsei Med J*. 53(1): 132–137.

****

OPEN ACCESS