
RESEARCH

THE MUTATION STATUS OF KRAS GENE CODON 12 AND 13 IN COLORECTAL ADENOCARCINOMA

(Status Mutasi Gen Kras Kodon 12 dan 13 di Adenocarcinoma Kolorektal)

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ABSTRAK

Kanker kolorektum merupakan salah satu kanker yang tersering di dunia. Target molekuler untuk pengobatan kanker kolorektum yaitu Epidermal Growth Factor Receptor (EGFR) dengan pemberian antibodi monoklonal anti-EGFR. Pemberian pengobatan ini tidak dapat memberikan efek dampak di pasien dengan status gen KRAS bentuk mutan, sehingga perlu dilakukan pemeriksaan status mutasi gen KRAS. Telitian berupa deskriptif dengan pendekatan potong lintang yang bertujuan untuk mendapatkan data status mutasi gen KRAS kodon 12 dan 13 di pasien adenocarcinoma colorectal. Deteksi mutasi KRAS dilakukan dengan teknik Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR RFLP) yang dikonfirmasi dengan sekuensing. Sampel telitian adalah 30 blok parafin yang diperoleh dari Rumah Sakit Dr. Soetomo Surabaya masa waktu Januari-Desember 2013. Setelah dilakukan ekstraksi DNA terdapat 21 sampel yang dapat digunakan untuk pemeriksaan lanjutan. Hasil PCR RFLP menunjukkan terdapat 7/21 mutasi pada kodon 12 dan tidak terdapat mutasi gen KRAS pada kodon 13. Mutasi pada kodon 12 yaitu GGT>GCT, GGT>GGA dan GGT>GAT yang menyebabkan perubahan asam amino Gly12Ala, Gly12Gly dan Gly12Asp. Simpulan telitian ini adalah mutasi gen KRAS kodon 12 pada adenocarcinoma colorectal di Rumah Sakit Dr. Soetomo Surabaya sebanyak 33%.

Kata kunci: Adenocarcinoma colorectal, PCR RFLP, KRAS

ABSTRACT

Colorectal cancer is one of the most common cancers in the world. The molecular target for treatment of colorectal cancer is epidermal growth factor receptor (EGFR) by anti-EGFR monoclonal antibody. Treatment with anti-EGFR antibody in patients with KRAS gene mutation had no effect, so it is necessary to check the mutation status of the KRAS gene. This study was a descriptive study with a cross sectional approach aimed to obtain the mutation status of KRAS gene in colorectal adenocarcinoma. Detection of KRAS mutations was done by using Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP PCR) confirmed by sequencing. The samples were 30 paraffin blocks obtained from the Dr. Soetomo Hospital during January-December 2013. After DNA extraction there were 21 samples that could be used for further examination. RFLP PCR Results showed 7/21 mutation in Kras gene codon 12 and no mutation in codon 13. Mutations in codon 12 were as follows GGT>GCT, GGT>GGA and GGT>GAT that changed amino acid Gly12Ala, Gly12Gly, and Gly12Asp. In conclusion, the mutation of codon 12 KRAS gene in colorectal adenocarcinoma at the Dr. Soetomo Hospital Surabaya was 33%.

Key words: Adenocarcinoma colorectal, PCR RFLP, KRAS

INTRODUCTION

Colorectal Cancer (CRC) is one of the most common cancers in the world. In 2013 approximately 102,480 cases of colon cancer and 40,340 cases of rectal cancer were diagnosed in the United States.¹ About 608,000 deaths from CRC are estimated worldwide,

accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer. The age-standardized incidence rates of CRC per 100.000 populations in Indonesia were 15.9 for males (the second most cancer after lung cancer for men) and 10.1 for females (the third most cancer after breast and cervix uterin cancer).²

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An important molecular target for the treatment of CRC is the Epidermal Growth Factor Receptor (EGFR) using monoclonal anti-EGFR antibody, cetuximab and panitumumab. Anti-EGFR binds to EGFR and phosphorylated EGFR to prevent and block ligand activation-induced EGFR tyrosine kinase, thereby preventing the activation of downstream signaling pathways PI3K and RAS/MEK/ERK. This results in cellular proliferation and induces barriers apoptosis.^{3,4}

Kirsten rat sarcoma-2 virus oncogene (KRAS) protein is one of the most important molecules in the EGFR downstream signaling pathway. KRAS gene, also called p21, encodes a small G-protein that functions as downstream of EGFR-induced cell signaling.^{4,5} When the EGF or other ligands occupy the EGFR, it activates a signaling cascade via several pathways, including the RAS-RAF-mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-AKT pathways, which control cell proliferation, differentiation and survival.⁵ RAS proteins normally cycle between active (RAS-GTP) and inactive (RAS-GDP) conformations. RAS proteins are activated by guanine nucleotide exchange factors (GEFs) which are recruited to protein complexes at the intracellular domain of activated receptors. Signaling is terminated when RAS-GTP is hydrolyzed to the RAS-GDP inactive complex by GTPase-activating proteins (GAPs). Under physiological conditions, levels of RAS-GTP in vivo are tightly controlled by the counterbalancing activities of GEFs and GAPs.⁶ Mutations in genes that encode RAS proteins disrupt this balance, causing perturbations in downstream signaling activities. These KRAS gene mutations result in RAS proteins that are constitutively in the active RAS-GTP conformation. Unlike wild-type RAS proteins which are deactivated after a short time, the mutated RAS proteins cause continuous activation of RAS signaling pathways in the absence of upstream stimulation of EGFR/HER receptors. This oncogenic activation of RAS signaling pathways has been implicated in many aspects of the malignant process, including abnormal cell growth, proliferation, and differentiation.⁶

The KRAS gene mutations occur in about 35-40% of CRC. This mutation is a single nucleotide point mutation in codons 12, 13 and 61. This mutation occurs during the development of CRC carcinogenesis. Approximately 17–25% of all cancers have mutations in the KRAS gene and 30–40% of CRC.⁷ KRAS gene mutations were found in exon 2 at codon 12 and codon 13 which is about 85-90% and at codon 61 (5%) and 146 (5%).⁴

The KRAS gene mutations have an impact on the selection of therapy in patients with CRC. Several studies have reported that KRAS mutations caused resistance to anti-EGFR monoclonal antibody. KRAS mutations were associated with poor response to therapy. KRAS gene mutations lead to the activation of downstream signaling pathways without stimulation upstream of the receptor EGFR/HER so that therapy with monoclonal anti-EGF antibody as a ligand of EGFR becomes useless.^{5,6} Although EGFR ligand bound with monoclonal anti-EGF antibody, activation of cell proliferation pathways continue so that the cell number is increasing. Based on this, it is necessary determine the status of the KRAS gene mutations in codons 12 and 13 in patients with colorectal adenocarcinoma in the Dr. Soetomo Hospital.

METHODS

This study was an observational study with cross sectional approach to determine the status of the KRAS gene mutations in patients with colorectal adenocarcinoma who underwent histopathologic examination at the Department of Pathology School of Medicine, Airlangga University-Dr. Soetomo Hospital in Surabaya from January to December 2013. The study has been approved by the Health Research Ethics Committee of the School of Medicine, Airlangga University No. 348/EC/KEPK/FKUA/2014.

The inclusion criteria of samples were histopathology examination conducted at the Department of Pathology Dr. Soetomo Hospital, microscopic appearance in accordance with the criteria of colorectal adenocarcinoma, and tissue in paraffin blocks which was still fairly representative for the purpose of this research. Exclusion criteria for samples was not enough tissue in paraffin blocks for research purposes.

Paraffin blocks with the inclusion criteria were taken for the DNA extraction randomly. The preparation process of paraffin blocks was conducted as previous studies.⁸ Paraffin blocks are cut to the size of 25 μm (5 μm \times 5 μm pieces). Microtome knife before being used to cut blocks of paraffin was washed with 20% chlorox bleach to avoid DNA contamination, then washed with detergent, rinsed with clean water, dried, and cleaned with absolute ethanol. Deparaffination was done by xylol and rehydration with ethanol. DNA extracted by QIAamp Tissue DNA Mini Kit (Qiagen) was done in accordance with the manual use of reagents and subsequently used as a template for Polymerase Chain Reaction (PCR).

Detection of KRAS gene mutations in codons 12 and 13 was performed by Restriction Fragment Length Polymorphism (RFLP) PCR as in a previous research. Primers used to detect mutations in codon 12, namely forward primer 5'-ACT GAA TAT AAA CTT GTA GTG GTT CCT GGA-3', reverse primer 5'-CTG TAT CAA AGA ATG GTC CAG CTG CAC TA-3' which produced 162 base pair (bp). Restrictions to determine the mutation of KRAS gene codon 12 used MvaI enzyme. The wildtype KRAS gene may be restricted to one location which produced two fragments, namely the size of 133 bp and 29 bp, while the mutant KRAS gene was not restricted resulting in a 162 bp fragment.⁹

Primers used to detect mutations in codon 13, namely forward primer 5'-GTA CTG GTG GAG GTG ATA TAT TAT TAA TTG-3' and reverse primer 5'-GTA AGG TCG TCA CAC CTA TGC TCT GG-3' that produced 159 bp. Restrictions to determine the mutation of KRAS gene codon 13 used BsuRI enzyme. The wildtype KRAS gene can be restricted in two locations producing three fragments of DNA which was measured 85 bp, 48 bp and 26 bp, while the mutant KRAS gene may be restricted to one location so as to produce two fragments which was measured 85 bp and 74 bp.⁹

The PCR composition were the master mix 10 μ L, dH₂O 5 μ L, forward primer 1 μ L, reverse primer 1 μ L, samples of 5 μ L. The PCR conditions were predenaturation at 94°C for 5 minutes, denaturation 94°C for 1 minute, annealing 63°C for 1 minute, extension 72°C for 30 seconds, 40 cycles and a final extension 72°C for 10 minutes. Electrophoresis used agarose 4% which was then visualized by gel documentation. PCR RFLP with positive results were confirmed by sequencing using ABI PRISM 310.

RESULTS AND DISCUSSION

In the year 2013, 139 colon and rectum neoplastic lesion were registered, with mean age (range 26–82 years old, males 74, females 65). The majority were histopathologically classified as carcinoma 85.61% and the rest was adenoma 5.03%, non-epithelial tumors 5.76%, and secondary tumours 3.6%.

This study used 30 paraffin blocks from patients aged 28–68 years. The most age was 41–50 years (9/30), age 51–60 years (7/30), age 61–70 years (6/30) and 31–40 years (6/30), age 28–30 years (2/30) of patients. Males were 18 and females were 12 patients.

The samples of this study used were the tumor tissues in the paraffin blocks. The tumor tissues were fixed with buffer formalin, and processed with formalin, alcohol, xylol, and paraffin, then the tumor tissues were embedded in the block of paraffin, called formalin fixed paraffin embedded (FFPE). Gold standard to analyze the Kras gene mutation is using specimen from the frozen tissue.¹⁰ However, routine procedures to diagnose the tumor tissue were performed by buffered formalin fixation, so that the tumor tissue was not stored in frozen tissue form but in the paraffin block. DNA extraction from FFPE samples had a poor quality of DNA.¹⁰ This was because the tumor tissues had undergone processing with the addition of alcohol, xylol, and paraffin blocks, causing fragmentation to DNA. This was consistent with the results of this research using 30 paraffin blocks but 9 blocks could not be used for the PCR process, so there were only 21 blocks that can be used for the detection of the KRAS gene mutations.

Identification of the KRAS gene mutation at codon 12 used primers KRAS 12F/R produced 162 bp and

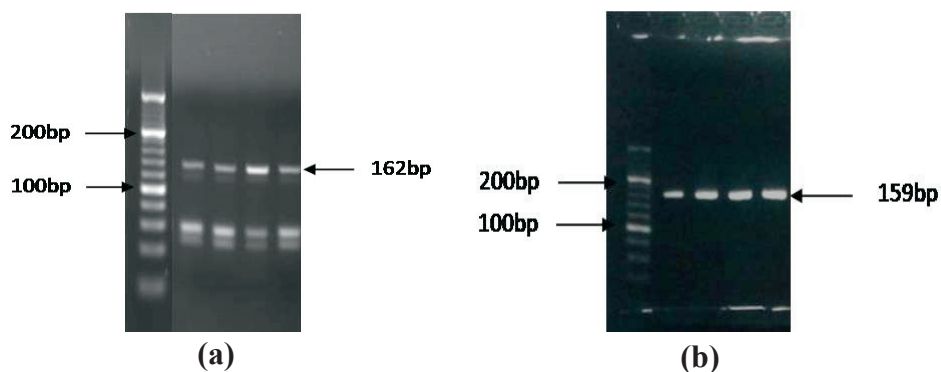


Figure 1. The identification of KRAS gene at codon 12 produced 162 bp (a) and codon 13 produced 159 bp (b).

KRAS 13F/R products 159 bp (Figure 1). Detection of KRAS gene mutations in this study was conducted by RFLP PCR, is a PCR technique to amplify DNA in PCR reaction, then the PCR product was restricted (cut) into multiple fragments of DNA using restriction enzymes. Restriction enzymes in this study used MvaI enzyme (also called BstNI) and BsuRI (also called HaeIII). Side restriction enzyme MvaI side was CCWGG, W was nucleotide A/T, while the restriction enzyme BsuRI was GGCC.

The PCR products were purified to clean the PCR products from the remaining PCR reagents so the restriction enzyme easily recognized the recognition sequences contained in the PCR product. After obtaining the pure DNA product from the codon 12 and 13, then restriction was carried out. Restriction reaction composition consisted of nuclease free water 16 μ L, 10 \times buffer R 2 μ L, 1 μ L pure DNA product and MvaI enzyme 1 μ L, then it was incubated at a temperature of 60°C for 16 hours. The restriction reaction product was then electrophorized using low melting agarose 4%.

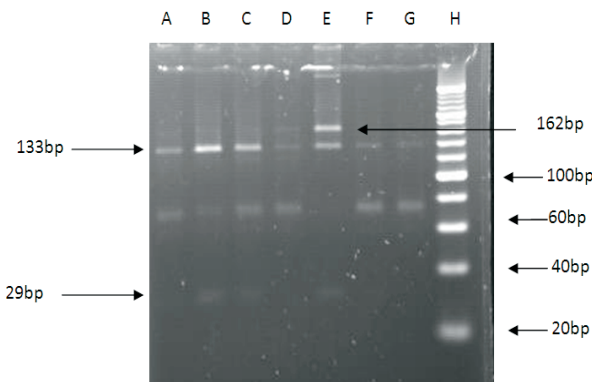


Figure 2. Restriction of the codon 12 PCR product with MvaI enzyme, producing 162 bp at the mutant type (D,E) and 133 bp, 29 bp at wild type (A,B,C,F,G) KRAS gene, H=marker.

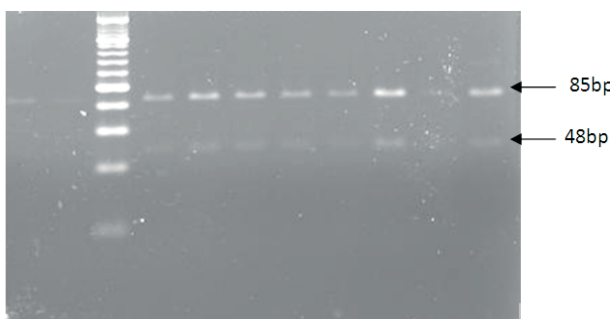


Figure 3. Restriction of the codon 13 PCR product with BsuRI enzyme, producing 85 bp, 48 bp and 26 bp at the wild type, 85 bp and 74 bp at the mutant type of KRAS gene.

The MvaI enzyme recognized the codon 12 PCR products at one location. If the wild type KRAS gene was restricted at one location resulting in two fragments of DNA with a size of 133 bp and 29 bp, if the mutant KRAS gene was not restricted, thus resulting in a 162 bp fragment (Figure 2, 4). The BsuRI enzyme recognized the codon 13 PCR product at two locations. The wild type KRAS gene was restricted at two locations resulting in three fragments of DNA with a size of 85 bp, 48 bp and 26 bp, while the mutant KRAS gene was then restricted at one location so as to produce 85 bp and 74 bp (Figures 3, 4).

The results of RFLP PCR with MvaI enzyme at codon 12 showed that the mutation of KRAS gene at

Table 1. The mutation status of KRAS gene at colorectal adenocarcinoma

No	Type	Tissue	The type of codon 12 mutation
1	Adenoca Well Diff	Rectosigmoid	GGT>GAT
2	Adenoca Well Diff	Colon	GGT>GCT
3	Mucinus Adenoca	Rectum	GGT>GGA
4	Adenoca Well Diff	Rectum	GGT>GGA
5	Adenoca Well Diff	Rectum	GGT>GAT
6	Adenoca Poor Diff	Anorectum	GGT>GAT
7	Adenoca Moderate Diff	Rectum	GGT>GCT

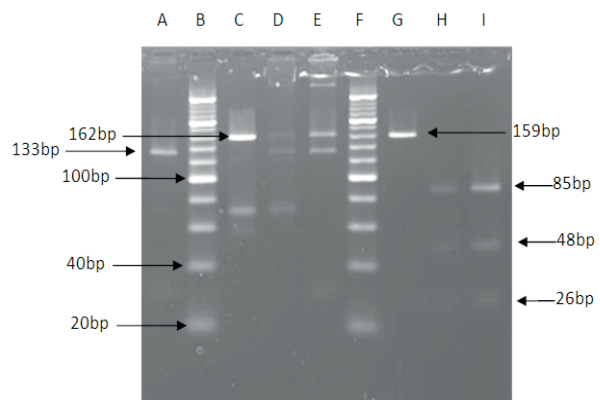


Figure 4. Restriction with MvaI at the codon 12 PCR product and BsuRI at the codon 13 PCR product. B, F=marker, A,C,D,E= the codon 12 PCR product, 162 bp (C), wildtype (A) and mutant type heterozygote (D,E) (mutant type produced band 162 bp, wild type produced band 133 bp and 29 bp, band 29 bp invisible). G,H,I = the codon 13 PCR product, 159bp (G), wildtype (H,I) (wild type produced band 85 bp, 48 bp and 26 bp, mutant type produced band 85 bp and 74 bp).

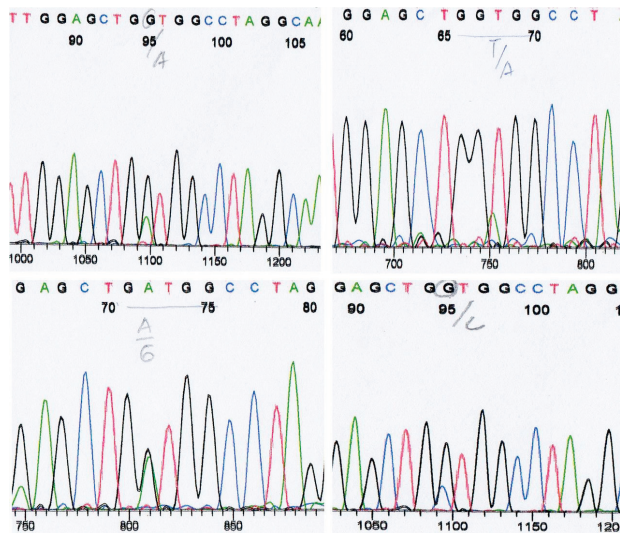


Figure 5. Part sequence of the mutant type of KRAS gene at GGT>GCT, GGT>GGA, GGT>GAT

codon 12 was found to be 33% (7/21) mutations and 67% (14/21) wild type. The restriction enzyme BsuRI at the codon 13 of KRAS gene showed that there was no mutation of KRAS gene. The KRAS gene at codon 13 in all samples in this study was a wild type (Table 1). The results were then confirmed by sequencing. The sequencing results also showed similar results with the results of RFLP PCR. Based on the results of sequencing it was known that mutations occurring at codon 12 were GGT>GAT, GGT>GCT and GGT>GGA (Figure 5). This mutation caused an amino acid change in Gly12Ala, Gly 12 Gly and Gly12Asp.¹¹

KRAS gene families include RAS (KRAS, NRAS, HRAS) located on chromosome 12, consisting of four exons and encodes 189 amino acid.¹⁰ KRAS gene encodes a protein guanosine-5-triphosphate (GTP)-binding which is an effector important for ligand-bound EGFR.⁴ Results of this study showed that mutation of the KRAS gene codon 12 were GGT>GAT, GGT>GCT, and GGT>GGA which caused amino acid changes Gly12Ala, Gly 12 Gly and Gly12Asp.

KRAS gene mutations in CRC were found in 30-40% of patients, that is 85-90% occurring in exon 2 codon 12 and 13.5% at codon 61 and codon 146.^{4,7} KRAS gene mutation was found in 31% of the 1989 cancer colorectal cases and this mutation was significantly associated with lower survival rates compared with wild type.¹² KRAS KRAS gene mutation was commonly found in the form of point mutations, G12D, G12A, G12R, G12C, G12S, G12V and G13D.¹¹ Mutation at codon 12, pG12D and pG12V was the most frequent, whereas mutations were often found at codon 13, pG13D.¹⁰ KRAS gene mutations in codons

12 and 13 play an important role in the progression of colorectal cancer and mutations in codons 12, 13 and 61 is a biomarker for lung cancer.⁶

These mutations impair the intrinsic GTPase activity of KRAS and prevent GAPs from promoting GTP hydrolysis by KRAS, therefore causing KRAS proteins to accumulate in the GTP-bound, active form. In this manner, mutant KRAS results in a constitutively active GTP-bound state and the activation of downstream pro-proliferative signaling pathways. Therefore, KRAS mutations play a critical role in human tumor genesis and are the most prevalent in pancreatic, thyroid, colorectal and lung cancers.¹⁰

The results of the research groups Kirsten Ras in the Colorectal Cancer Collaborative Group Study (RASCAL Study)¹² of 2,721 patients with colorectal cancer who came from 13 countries, showed that KRAS gene cases increases the risk of recurrence and death, especially mutation guanine (G) into Thymine (T). Such mutations are not associated with sex, age, large tumor, or Dukes' stage. Type of cancer with poorly differentiated rarely mutated KRAS. RASCAL II Study revealed that KRAS mutations occur in the development of pre-cancerous adenomas in the colon and rectum. Mutation glycine to valine becomes important to determine the progression of cancer and also predispose to cancer to become more aggressive evolved into advanced colorectal cancer.¹³

KRAS mutations have also been associated with more rapid and aggressive metastatic behavior of colorectal liver metastases. KRAS mutation and high Ki-67 expression were associated with multiple liver metastases, shorter time interval to their detection; and with poor survival after colon resection. Additionally, KRAS mutation was independently associated with poor survival after liver resection. KRAS C12V mutations were more frequently associated with hepatic metastasis. Further, in a recent study of 143 Korean patients with metastatic or recurrent colorectal cancer, lung metastasis was more frequently the initial metastatic site in patients with the KRAS mutations.⁷

There is evidence in multiple randomized trials of improved response rate, progression free survival and/or overall survival in response to anti-EGFR MoAb therapy only in patients with no mutations in codon 12 or 13 versus mutated KRAS tumors. Since then, KRAS mutations have emerged as a major predictor of resistance to EGFR inhibitors in first-line as well as in subsequent lines of treatment. The American Society of Clinical Oncology (ASCO) recently published their provisional clinical opinion that patients with mCRC, having a KRAS mutation in codon 12 or 13, should not receive anti-EGFR antibody treatment.⁷

CONCLUSION AND SUGGESTION

The conclusion of this study is that the KRAS gene mutation from paraffin blocks of the colon and rectum adenocarcinoma patients in the Dr. Soetomo Hospital obtained 33% (7/21), which occurred at codon 12 is GGT>GCT, GGT>GGA and GGT>GAT causing amino acid changes Gly12Ala, Gly12Gly and Gly12Asp and there was no mutation at codon 13.

Suggestion of this study is the identification of the KRAS gene mutation status can be performed on all types of malignancy of the tumor solid tissues to determine the targeted therapy of malignancy.

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REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer Statistics, 2013. *Ca Cancer J Clin* 2013; 63(1): 11–30.
2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: <http://globocan.iarc.fr>; accessed on 20/July/2016.
3. Dobre M, Comanescu M, Arsene D, Iosif C, Bussolati G. K-ras gene mutation status in colorectal cancer comparative analysis of pyrosequencing and PCR-RFLP. *Rom J Morphol Embryol*. 2013; 54(3): 567–574.
4. De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol*. 2011; 12(6): 594–603.
5. Prenen H, Tejpar S, Van Cutsem E. New Strategies for Treatment of KRAS Metastatic Colorectal Cancer. *Clin Cancer Res*. 2010; 16(11): 2921–2926.
6. van Krieken JHJM, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, Quirke P, Flejou JF, Plato Hansen T, de Hertogh G, Jares P, Langner C, Hoefler G, Ligtenberg M, Tinialos D, Tejpar S, Bevilacqua G, Ensari A. KRAS mutations for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch*. 2008; 453(5): 417–31.
7. Arrington AK, Heinrich EL, Lee W, Duldulao M, Patel S, Sanchez J, Garcia-Aguilar J, Kim J. Prognostic and Predictive Roles of KRAS Mutation in Colorectal Cancer. *Int J Mol Sci*. 2012; 13(10): 12153–68.
8. Siriaunkgul S, Utaipat U, Suthipintawong C, Tungsinnmunkong K, Triratanachat S, Khunamornpong S. HPV Genotyping an adenocarcinoma of the uterine cervix in Thailand. *Int J Gynaecol Obstet*. 2013; 123(3): 226–30.
9. Hatzaki A, Razi E, Anagnostopoulou K, Iliadis K, Kodaxis A, Papaianou D, Labropoulos S, Vasilaki M, Kosmidis P, Saetta A, Mihalatos M, Nasioulas G. A Modified Mutagenic PCR RFLP Method for K-ras Codon 12 and 13 Mutation Detection in NSCLC Patients, *Molecular and Cellular Probes*. 2001; 15(5): 243–7.
10. Tan C, Du X. KRAS mutation testing in metastatic colorectal cancer. *World J Gastroenterol*. 2012; 18(37): 5171–80.
11. Gonzalez de Castro D, Angulo B, Gomez B, Mair D, Martinez R, Suarez-Gauthier A, Shieh F, Velez M, Brophy VH, Lawrence HJ, Lopez-Rios F. A Comparison of Three Methods for Detecting KRAS Mutations in Formalin Fixed Colorectal Cancer Specimens. *British Journal of Cancer*. 2012; 107(2): 345–51.
12. Phipps AI, Buchanan DD, Makar KW, Win AK, Baron JA, Lindor NM, Potter JD, and Newcomb PA. KRAS-mutation status in relation to colorectal cancer survival: the joint impact of correlated tumour markers. *British Journal of Cancer*. 2013; 108(8): 1757–64.
13. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter “RASCAL” study. *J Natl Cancer Inst*. 1998 May 6; 90(9): 675–4.
14. Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, Young J, Walsh T, Ward R, Hawkins N, Beranek M, Jandik P, Benamouzig R, Jullian E, Laurent-Puig P, Olschwang S, Muller O, Hoffmann I, Rabes HM, Zietz C, Troungos C, Valavanis C, Yuen ST, Ho JW, Croke CT, O’Donoghue DP, Giaretti W, Rapallo A, Russo A, Bazan V, Tanaka M, Omura K, Azuma T, Ohkusa T, Fujimori T, Ono Y, Pauly M, Faber C, Glaesener R, de Goeij AF, Arends JW, Andersen SN, Lövig T, Breivik J, Gaudernack G, Clausen OP, De Angelis PD, Meling GI, Rognum TO, Smith R, Goh HS, Font A, Rosell R, Sun XF, Zhang H, Benhattar J, Losi L, Lee JQ, Wang ST, Clarke PA, Bell S, Quirke P, Bubb VJ, Piris J, Cruickshank NR, Morton D, Fox JC, Al-Mulla F, Lees N, Hall CN, Snary D, Wilkinson K, Dillon D, Costa J, Pricolo VE, Finkelstein SD, Thebo JS, Senagore AJ, Halter SA, Wadler S, Malik S, Krtolica K, Urošević N. Kirsten ras mutations in patients with colorectal cancer: the ‘RASCAL II’ study. *Br J Cancer*. 2001 Sep 1; 85(5): 692–6.