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Evaluating the Superoxide Dismutase-1 status in Wild Type and Mutant at codons 12 and 13 of KRAS gene Spectrum for the Patients with Sporadic Colorectal Cancer

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Abstract: KRAS gene is involved in G protein- mediated signal transduction pathway and has constitutive GTPase activity, which is a loss when the gene is mutated. It was reported that mutation in KRAS codons 12 and 13 are the most frequently detected mutation (hot spots) in human colorectal cancers. SOD-1 is an enzyme that in humans is encoded by the SOD1 gene, located on chromosome 21 and it is one of three human superoxide dismutases. The aim of the current study was to evaluate the role of wild-type and mutant genotyping of KRAS gene on the SOD-1 levels and its related trace elements (copper and zinc) in patient with sporadic colorectal cancer who received adjuvant chemotherapy regimen. This investigate was done by extraction of DNA from whole blood and used PCR-RFLP technique to determine the different genotypes of KRAS gene. SOD-1 concentration was assessed by competitive ELISA method. The results of this study showed the frequency of WT and mutant alleles of KRAS gene were 61.5% and 38.5%, respectively. Also, the results showed significant decreasing in the levels of SOD-1 in mutant status of patients with sporadic CRC compare to WT and control group ($p<0.05$).
Keywords: Sporadic colorectal cancer, KRAS gene, SOD-1, wild-type, mutant alleles.

Introduction

Colorectal cancer (CRC) is one of the most frequent malignant and lethal diseases in the developed countries. CRC is a general term that is used to describe cancer occurs in both colon and rectum[(1)]. Iraqi cancer board reported that CRC is the seventh most common cancer in Babylon Province[(2)]. The expected change in the pattern of this disease in Iraqi is related to the rapid change in dietary habits[(3)]. The development of CRC either as a part of a hereditary cancer syndrome, or sporadically is induced by inflammatory bowel disease[(4)]. Sporadic carcinomas devoid any familial or inherited predisposition accounts for approximately 90% of CRC[(5)]. KRAS is one of the most repeatedly mutated oncogenes in CRC. Mutant KRAS is present in approximately 40-50% of CRCs. It is considered to play an important role in the relatively early stages of colon and rectum carcinogenesis[(6)]. KRAS is located on the short arm of chromosome 12 (at position 12.1). The total genomic size of KRAS is 45675 base pairs with 5 exons (only 4 coding) and coded of RAS protein[(7)]. Generally, RAS protein is activated in response to extracellular stimuli including a growth factor, EGF, platelet-derived growth factor, granulocyte-macrophage colony-stimulating factor,.. etc. It has been observed that under normal conditions inactive cells in Go, have less than 5 % of their total RAS protein in the active state compared with nearly 50 % upon mitogenic stimulation. So, RAS depending signaling, mediates proliferation or

differentiation([8]). Adjei *et al* 2001[9] reported that Ras-mediated activation of the MEK/MAP kinase pathway is one of the factors which raises cellular levels of cyclin D1. The increase levels of cyclin D1 promotes the progression of cells through G1check point and into S phase, thus leading to proliferation[10]. Following EGF binding to its receptor and activation of tyrosine kinases, the RAS protein becomes activated by binding to GTP, transduction the activation signal to the nucleus by MAPKs and PI3K/AKT-mediated cascades[11]. Specifically, the active state of the RAS protein is facilitated by binding to the Grb2 protein, which interacts with the SH3 domains of the SOS protein, a member of the nucleotide exchange factor family. In the GTP state, RAS is able to activate downstream proteins and to regulate cell transformation[12]. Superoxide dismutase (SOD) is the antioxidant enzyme catalyzes the reaction between two superoxide radicals (O_2^-) to yield oxygen and hydrogen peroxide H_2O_2[13] . The superoxide reacts rapidly with the nitric oxide free radical $NO^{\cdot}$ and forms a reactive species of nitrogen, called peroxynitrite, causing damages in the cellular DNA as well as induces the decoupling of eNOS, which leads to a higher production of superoxide maintaining the conditions of endothelial damage[14]. SOD, a major antioxidant defend against oxidative stress by preventing toxic effects of superoxide[15].

Aims of the study

Investigate the relationship between age, sex, cancer stages, cancer site, chemotherapy status, obesity, and smoking status with the frequency of wild-type and mutant alleles of KRAS genes in sporadic colorectal cancer patients. The other aim was to evaluate the SOD-1 levels in the wild-type and mutant genotyping of KRAS gene of a patient with sporadic colorectal cancer who received adjuvant chemotherapy regimen.

Material and methods

This study was performed at the laboratories of Biochemistry Department, College of Medicine, University of Babylon. The collection of samples was conducted during the period from 1st of March 2014 till 30th of June 2015. The patients group who subjected in this study were (52) patients in the age group ranging from 39 - 75 years, the mean ± standard deviation (SD) was (59.3 ± 10.68 years). All of those patients were screened and treated with adjuvant chemotherapy in the oncology centre of Merjan Teaching Hospital in Babylon province with clinical symptoms of colorectal cancer. All patients classified into four Dukes stages (A, B, C, and D) depending on TNM of the histopathological state. Fifty-two apparently healthy individuals (without gastrointestinal diseases) were taken as a control group with the age ranging from 37-75 years, the mean ± SD was (57.8 ± 11.1 years).

1-PCR-RFLP analysis for codons 12 and 13 of KRAS gene

Amplification of exon 1 codon 12 and 13 of KRAS gene was done by using the following primers, as shown in Table (1)[16].

Table (1):A primers with optimal annealing temperature used to amplification of KRAS gene codon 12 and 13 for PCR-RFLP analysis.

PPRIMERS F+R(5′ - 3′)	C CODON	ANNEALING TEMP.	AAMPLICON LENGTH
FF: ACTGAATATAAACTTGTGGTAGTTG GACCT R R: TAATATGTCGACAAAACAAGAT TTACCTC	1 12	55c^{o}	1 135 b
FF: GTACTGGTGGAGTATTTGATGTGT ATTAA RR: GTATCGTCAAGGCACTCTTGCCT AGG	1 13	50 c^{o}	1 159bp

Table (2): Amplification conditions of codon 12 and 13 of KRAS gene

Cycles	Function	Time(MIN)	Temp.(C°)	Stage
	Initial denaturation	5	95	1
	DNA denaturation	1	95	2
40	Primer annealing	1	55 / codon 12 50 /codon 13	
	Template elongation	1	72	
	Final elongation	7	72	3
Hold	Incubation	-	8	4

Then, the amplification products separated by electrophoresis through 1.5% agarose gel stained with ethidium bromide. The PCR product was digested with the restriction enzymes depending on the methods that described by *MvaI* (Micrococcus varians)[17] restriction enzyme used for codon 12 product (135bp) and *HaeIII* (Haemophilus aegyptius) for codon 13 product (159bp) (restriction endonuclease).

2- Determination of SOD-1 level

Human SOD-1 assay was based on standard competitive enzyme-linked immune-sorbent assay technology(ELISA) kit. This kit was provided from Elabscience®/ (cat# E-EL-H1113) and the assay performed depending on the manufactured instructions.

Results

KRAS codon 12 and 13 polymorphism analysis

The process of PCR-RFLP for KRAS gene codon 12 by restriction enzyme (*MvaI*) resulted in a products with two bands 106 and 29 bp for normal DNA (wild-type) and a single band 135bp for mutant and the products with three bands 135, 106, and 29 bp for mutant DNA of patients (mutant case has both allele, normal and mutant), as shown in Fig. (1)

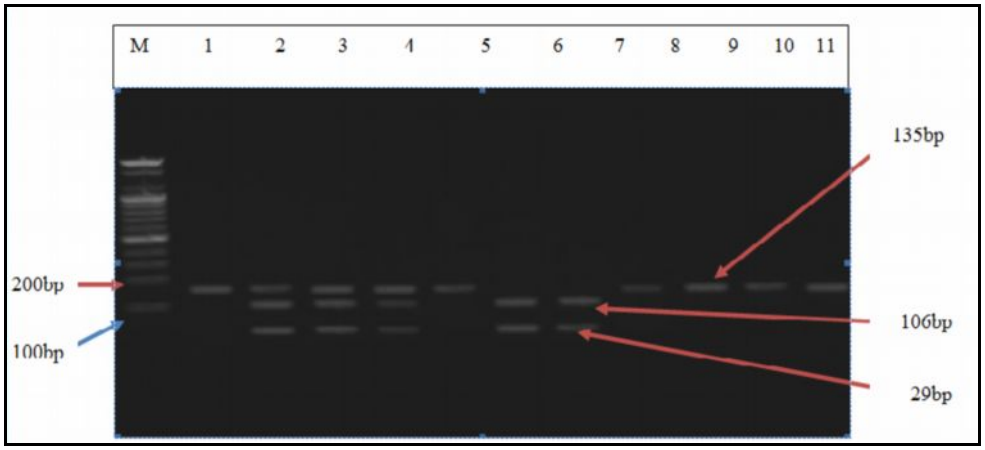
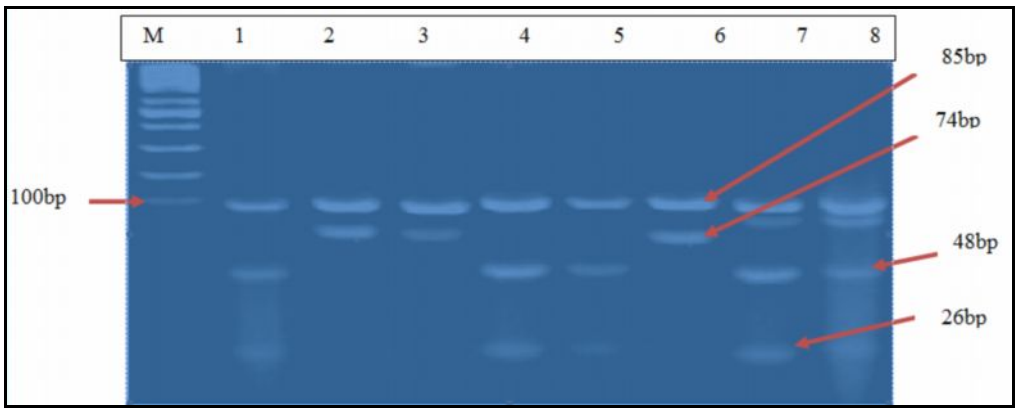


Figure (1) : Electrophoretic picture (agarose) represents the KRAS genotyping codon 12 , where lane M is 100 bp DNA ladders, lanes (2,3,4) were cases with both alleles mutant and normal (135bp+106bp+29bp), lane (1,5,8,9,10,11) mutant (135bp) and, lanes (6,7) are wild-type (106bp+29bp)(normal) allele.

For codon 13 of KRAS gene, (*HaeIII*) restriction enzyme cleavage of 159bp of PCR product (amplicon) into three bands as wild-type allele (85+48+26bp). The mutant allele was cleavage by RE into two bands(85+74bp), as shown in Fig.(2).



Figure(2): Electrophoretic picture (PAGE) represents the KRAS genotyping codon13 , where lane M is 100 bp DNA ladders , lane (1,4,5) represent of wild-type allele, lane (2,3,6) represent of mutant allele, and lane (7,8) represent cases have both, normal and mutant allele.(PAGE: Polyaceylamide gel electrophoresis 6%).

Figure (3) summarizes the frequency distribution of wild-type and mutant of study patients according to KRAS gene codon 12 and 13 polymorphism.

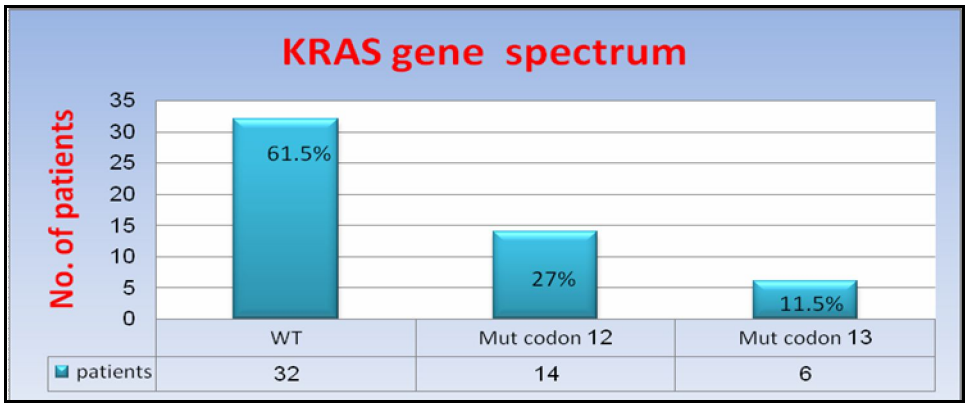


Figure (3): Frequency of the detected KRAS mutations and wild-type in patients group.

The frequencies of KRAS codon 12 and 13 polymorphism (WT and Mutant) in patients group and it correlation with age, gender, cancer stage, cancer site, and chemotherapy status were tested by Fisher´s exact test, as summarized in Table (3).

Figure (3) summarizes the frequency distribution of wild-type and mutant of study patients according to KRAS gene codon 12 and 13 polymorphism. Out of 52 cases, 20(38.5%) cases were mutant and 32(61.5%) were wild-type. 14(70%) of mutant cases were detected in codon 12 and only 6(30%) mutant cases were detected in codon 13. The frequency of mutation in codon 12 and 13 was 27% and 11.5% respectively.

Table (3): Relationship between KRAS gene WT and mutant with clinic-pathological variables in sporadic CRC patients.

VARIABLES	WT N=32 (61.5)	MUT CODON 12 N=14 (27)	MUT CODON 13 N=6 (11.5)	P-VALUE
< 50 N=22 ≥ 50 N=30	17(77) 15(50)	3(14) 11(37)	2(9) 4(13)	0.142
Male N=30 Female N=22	18(60) 14(64)	9(30) 5(23)	3(10) 3(13)	0.844
A+B N=19 C+D N=33	14(74) 18(55)	1(5) 13(39)	4(21) 2(6)	0.008*
Colon N=37 Rectum N=15	22(59) 10(66)	13(35) 1(6)	2(6) 4(29)	0.022*
Half dosage N=30 Total dosage N=22	19(63) 13(59)	9(30) 5(23)	2(7) 4(18)	0.547
Never N=22 Ever N=30	18(82) 14(47)	3(14) 11(36)	1(4) 5(17)	0.046*
Over weight N=24 Normal weight N=28	14(58) 18(64)	8(33) 6(22)	2(9) 4(14)	0.656

Figure (4) is shown the levels of SOD-1(pg/ml) in control group compare to patients (WT, mutant codon 12, and mutant codon 13).

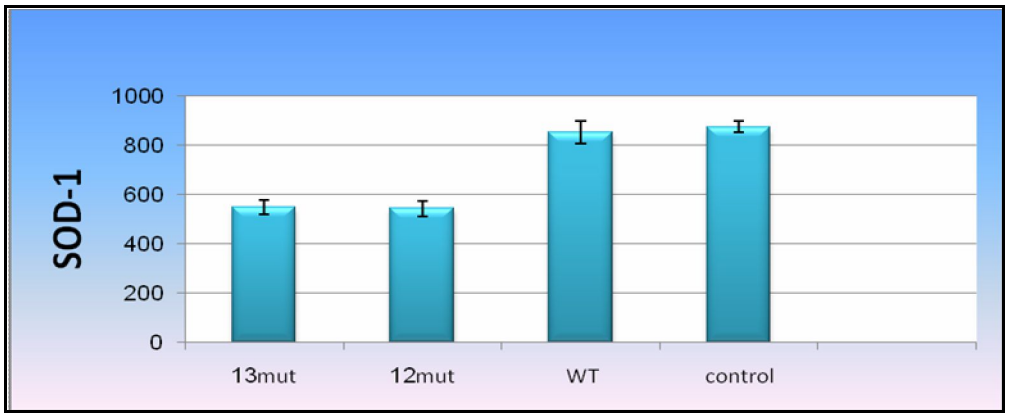


Figure (4): Mean ±SD of SOD-1 of patients (WT and mutant) compare to control group.

Discussion

KRAS gene is an oncogene that encodes for the RAS protein, which is a small membrane-bound G protein. RAS protein is activated by receptor tyrosine kinases, and it plays a crucial role in the regulation of cell division by transferring external proliferation signals to the nucleus. Ras proteins control signaling pathways that are key regulators of several aspects of normal cell growth and malignant transformation.. Activating

KRAS gene mutations have been detected in approximately 35-50% of CRCs, and these mutations are associated with poor therapeutic responses[18]. The analysis of KRAS mutation spectrum is, therefore, very important for the adequate treatment of CRC patients in Babylon/Iraq, as the methodology adopted in the present study for detecting the mutation can be used for diagnostic purposes in future. The results of present study suggests that the frequency of mutation that occurs in KRAS gene in patients with sporadic colorectal cancer in Babylon populations was 38.5% and this in agreement with published studies in different areas on the world[19,20].There was a moderate frequencies of KRAS mutation was recorded in the present study in Babylon populations (38.5%). High mutation frequency of KRAS gene has been observed in colorectal adenomas , in addition the mutation found could be lost (become moderate) through cell progress from adenoma to carcinoma[21]. KRAS mutation may be a common early event in carcinogenesis and also that the etiological factors for sporadic CRC in Babylon are likely to be different. Mutational spectrum of KRAS has been widely studied in western countries whereas no data is available on the spectrum of mutations for Babylon populations. The mutated protein is locked in the active form due to impaired GTPase activity, which hydrolyses GTP to GDP[22]. Ras activation affects multiple cellular pathways that control cellular growth, differentiation, survival, apoptosis, cytoskeleton organization, cell motility, proliferation, and inflammation[23]. Also the moderate frequency mutation in KRAS gene that occurs in early events of incidence with CRC in Babylon population may be need further studies with large sample size to support the results of the present study. Table (3.13) showing patients with wild type (63vs.59%) and codon 12(30vs.23%) mutations not derived significant benefit from chemotherapy, while those with codon 13(7 vs. 18%) mutations got limit benefit but not significant and this in agreement with the previous published studies[24-29]. Table (3.14) showed high frequency and significant difference in the incidence of the mutation in KRAS gene codon 12 in advanced stages C+D compared to early stages A+B(39vrs5%)($p<0.05$). The higher frequency of mutation in KRAS gene codon 12 in Duke's C+D (39%) compared with Duke's A+B (5%) suggests that these mutations are associated with a more aggressive phenotype[30]. Furthermore, comparison of the mutation spectrum of the present study with other studies[31-35] showed both similarities and differences in overall of KRAS mutation frequency, no correlation with age, gender, chemotherapy status, and obesity status. The moderate incidence of KRAS mutation of sporadic colorectal cancer patients in the present study population may be strange. Only one study suggested the frequency of KRAS mutation in Iraq population was 48% [36]. The results of this study showed does not statistically deference between the levels of SOD-1 in wild-type subgroup compare to control ($p>0.05$) , but the results also showed highly significant difference of SOD-1 levels between wild-type compare to mutant codon 12 and 13 subgroups ($p<0.05$). It was reported that activating mutations in the KRAS were associated to the occurrence of mitochondrial dysfunction, and increase of aerobic glycolysis and of intracellular levels of reactive oxygen species (ROS)[37]. Evidences indicate that the increasing of ROS induced KRAS (Mut.) is functionally relevant to the malignant transformation, but excessive amounts of ROS cause oxidative consumption antioxidant agents and injuries to the cell [38]. While high levels of ROS production may leads to the induction of apoptosis or necrosis, increasing evidence demonstrates that low or transient ROS exposure increases cell proliferation, likely through altered expression of growth factors and proto-oncogenes[39-41]. ROS can stimulate signal transduction pathways and lead to activation of key transcription factors such as Nrf2. Recent studies demonstrated that KRAS G12D (normal gene) induces maintenance of low intracellular ROS levels via the transcription factor Nrf 2 (nuclear factor 2), which is a master switch in the antioxidant network[42]. Low levels of Nrf2 or loss of Nrf2 activity appears to increase ROS production and DNA damage and predisposes cells to tumorigenesis[43,44]. In result, the mutations in KRAS gene leads to changing in amino acids sequences (188 amino acids of p21 protein) and this may leads to unregulated in downstream cell-growth and exit in an active status (active cascade) and induce decreasing in the synthesis of some enzymatic antioxidant such as SOD-1.

Conflicts of interest

The authors have no conflicts of interest to declare in relation to this research.

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