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ASSESSING THE EXPRESSION OF BRAF GENE IN PARAFFIN-EMBEDDED BLOCKS OF PATIENTS WITH COLORECTAL CANCER

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ABSTRACT

Colorectal cancer is the second cancer related deaths in the world. Understanding the molecular pathway of that can provide some useful information about therapeutic manners. Hyper activity of BRAF gene has been reported in recent years and it can be proposed as a diagnostic molecular marker in many cancers. The purpose of this study was to assess the expression of BRAF gene in paraffin-embedded blocks of patients with colorectal cancer. In this study, 5 samples of paraffin-embedded blocks which were from a middle age patients with a sample of normal person were collected. After sectioning and paraffin removal, RNA was extracted and then cDNA synthesis was performed by using MMULV enzyme, Oligo dt and random hexamer primers. BRAF specific primers and β-actin (as an internal control) were extracted from high cited articles. RT-PCR reaction results indicated the increased expression of BRAF gene in carcinogenic cancer compared with normal sample. The results suggest the higher expression of BRAF gene in patient with colorectal cancer. Investigating the increased expression of BRAF gene in paraffin-embedded samples can be considered as an appropriate manner for research on old samples in hospitals and scientific institutes.

Key words: colorectal cancer, BRAF, Paraffin-embedded blocks, RT-PCRR

INTRODUCTION

Many people lose their lives annually. Colorectal cancer is the third common cancer in Iranian men and the forth one in Iranian women[1]. BRAF, a member of the RAF family, is a protein kinase that is encoded by the *BRAF* gene. The RAF family of proteins includes isoforms isoforms: ARAF, BRAF, and CRAF. While each isoform plays a role in the RAS-RAF pathway, BRAF is the main activator of MEK. BRAF plays an important role as an intermediary in the RAS-RAF signaling cascade, a pathway responsible for normal cell growth, differentiation, and survival.Preclinical studies demonstrate that mutations in the *BRAF* gene allow for BRAF to signal independently of upstream cues. As a result, mutated BRAF causes overactive downstream signaling via MEK and ERK. This leads to excessive cell proliferation and survival, independent of growth factors[2-4].Approximately 30% to 50% of colorectal tumors are known to have a mutated (abnormal) *KRAS* gene, However, 40% to 60% of patients with wild-type *KRAS* tumors do not respond to such therapy[6]. In these patients, data suggest that the mutated *BRAF* gene, which is present in 5% to 10% of tumors, can affect response to these agents[7-9].BRAF is a member of the RAF kinase family of growth signal transduction protein kinases. This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion[10]. More than 30 mutations of the *BRAF* gene associated with human cancers have been identified. The frequency of BRAF mutations varies widely in human cancers, from more than 80% in melanomas and nevi, to as little as 0–18% in other tumors, such as 1–3% in lung cancers and 5% in colorectal cancer[11]. many studies have done about the RAF kinase family in fresh blood samples, but by Considering the importance of using the paraffin-embedded samples in studying different cancers in statistically researches, it seems to be necessary to diagnose different markers in paraffin-embedded blocks. Due to the high incidence of colon cancer and the importance of statistical researches during several years, the evaluation of BRAF expression in old paraffin samples can be an effective step in statistical researches. Until now, there were no studies on evaluating the expression

MATERIALS AND METHODS

RNA extraction by RNX-PLUS solution (Sina gene- Iran):

In this study 6 paraffin blocks (1 patient, 1 normal) were selected for sectioning by pathologist. Age range of sample were 57 years old and samples were collectedin 2013.after sectioning the samples by microtome, paraffin was removed by xylene and alchol 75%.1000 µl of RNX-PLUS

were added and resulted solutions were vortexed for 5 seconds. Tubes were placed at room temperature for 5 minutes, then 200 µl of chloroform was added into each tube. After 15 seconds of vortex, tubes were incubated on ice for 5 minutes. Then they were centrifuged for 15 minutes at 13000 RPM. Supernatant fluid was transferred to a new tube and isopropanol was added with the same volume and then they were inverted in the tube 10 times. The tube was maintained for 6 hours at -20°c and thenthey were centrifuged for 15 minutes at 13000 RPM and the supernatant fluid was slowly drained. 1 ml of ethanol 75% was added to the pellet and they were inverted 10 times. Again they were centrifuged in 7500 rpm for 8 minutes. After draining the supernatant, pellet was dried and sediments were dissolved in DEPC water and then stored in -80°c.

CDNA Synthesis:

Vivantis kit (2 step RT-PCR kit) was used for cDNA synthesis. cDNA synthesis was performed according to kit instructions. Initially 10 µl of RNA, 1 µl of 10 Mm DNTP and 1 µl of random hexamer were mixed together. Then they were placed for 5 minutes in 65°C. Then 0.5 ml MMULV and 2 µl of 10X MMULV buffer were added to each reaction. Eventually the final volume reached to 20 µl by adding water. The tube was incubated for 1 hour at 42°c.

Specific primers for RT-PCR:

B-actin gene was used as an internal control. Its sequencing of forward and reverse primers were extracted from an article which were written by Berletch et al[12] and the primers of B-ACTIN gene were extracted from the article which were written by JB Berletch et al[13]. table 1 shows the Sequencing of β-actin and BRAF primers.

Table 1: characteristics of primers:

Name	Sequences	Molecular weight
BRAF-F	**CCCGGCTCTCGGTTATAAGA**	**195 bp**
BRAF-R	**TGGGCAGGAAGACTCTAACG**	
β-ACTIN F	**AGAGCTACGAGCTGCCTGAC**	**395 bp**
β-ACTIN R	**AGCACTGTGTTGGCGTACAG**	

RT-PCR (Reverse Transcriptase PCR)

Each reaction contains 12 µl cDNA template (Prepared cDN), 2.5 µl of 10X PCR buffer, 1 µl of each forward and reverse primers, 0.4 µl of 50 mM $Mgcl_2$, 0.5 µl of 10 mM dNTP (dTTP , dGTP , dCTP, dATP) and 0.4 µl of tag DNA polymerase.The final reaction was increased to 25 µl. thermal protocol for initial denaturation was 3 minutes at 95°c in 30 seconds for denaturation, 69°c in 30 seconds for annealing (β-actin) and 67°c for BRAF, 72°c in 50 seconds for extension and finally 72°c in 5 minutes as the final extension.

RESULTS

After optimization of RT-PCT by gene specific primers, electrophoresis of amplified sequences was done in 1.5% agarose gel containing SYBR (Sina gene- IRAN) in 0.5X TBE buffer. β-actin gene was used as internal control. Since the expression of these kinds of genes is permanent in cells, β-actin is considered to be a good option for examination of internal control. Results of β-actin as an internal control gene were positive in all tests which indicated the accuracy of the method used for RNA extraction and it was also played a confirmatory role in cDNA synthesis. The expression of BRAFwas observed in all carcinogenic paraffin-embedded block samples and there was no significant band in healthy sample.The results are shown in diagram1 and Figure1.

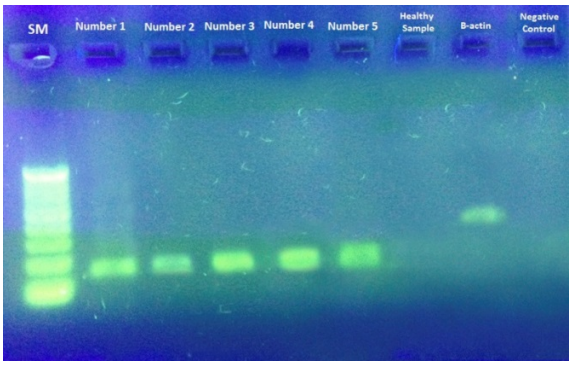

Figure1: not only Amplified sequences of BRAF gene was observed in cancer samples but also B-actin gene expression was observed. No expression was observed in negative control and healthy sample.

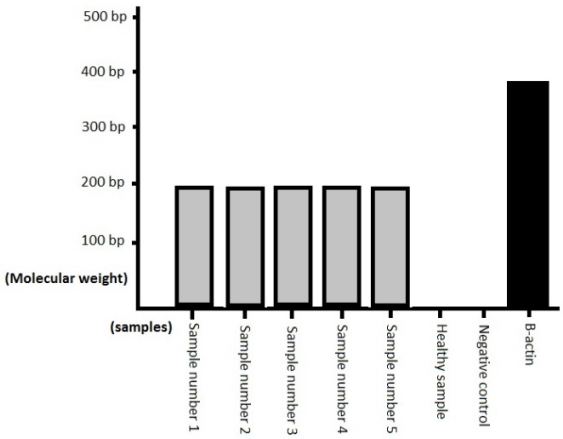

Diagram1: All cancerous samples had significant expression of BRAF gene and no expression was seen in negative control and healthy samples. The expression of β-actin confirmed the correctness of RNA extraction and cDNA synthesis progresses.

DISCUSSION

Colorectal cancer is one of the most common cancers worldwide. About 40-50% of newly diagnosed patients are affected with metastatic disease and the average survival of patients is between 18-21 months[14.15]. The detection of BRAF mutations is currently included in some clinical laboratory

protocols, although it has not been established as routine clinical practice. BRAF is a protein member of the RAF family (RAF1, BRAF, ARAF), also regulated by RAS binding. BRAF encodes a serine-threonine protein kinase that is the most important downstream effector of activated KRAS[16]. Mutated BRAF activates a signaling cascade involving proteins in the mitogen-activated protein kinase system, resulting in cell proliferation[17]. Approximately 15% of CRCs have the BRAF mutation, and this is an indicator of poor prognosis regardless of the treatment or administration [18]. Most of the BRAF mutations associated with cancer are located in exons 11 and 15, coding for the kinase domain. The hotspot mutation is the T-to-A transversion at nucleotide 1796 that corresponds to the V600E mutation. This mutation is predisposed to the inhibition of apoptosis and also aids in increasing invasiveness[19]. It has also been suggested that BRAF mutation is a negative prognostic indicator in CRC[20] and a negative predictor of response to EGFR inhibitors, according to results from CRYSTAL, OPUS, and PICCOLO trials [21-23]. BRAF mutation was also associated with shorter progression-free survival (PFS) and overall survival(OS)[24-26].KRAS and BRAF mutations are mutually exclusive in CRC[26.27] therefore, the National Comprehensive Cancer Network (NCCN) suggests considering BRAF mutation testing when KRAS is wild-type[28]. Different studies demonstrated that BRAF mutation confers resistance to both cetuximab and panitumumab[27]. Specifically, BRAF is responsible for resistance when patients received anti-EGFR therapy in a second or subsequent round of treatment, as shown in several retrospective studies [27.29.30.31]. In contrast, the predictive value of BRAF mutations in first line treatment has not been fully demonstrated [20.32.33]. A recent study conducted by Saridaki et al. showed lower PFS and OS in BRAF V600E mutated patients compared with wild-type (4.2 versus 11.1 months and 14.3 versus 35.0 months, resp.), although differences were not significantly significant [33]. Due to the poor prognosis of BRAF mutated patients and the lack of response to anti-EGFR therapy, rational therapeutic strategies have been directed toward selective RAF inhibitors. For instance, BRAF inhibitors used for melanoma have also been tested against CRC. However, very little clinical benefit was observed, suggesting that the biological behavior in melanoma and colorectal cancer can be different. The techniques which used in most studies were protein identification

bases techniques. And also serology methods (because of containing false positive and negative results) are not very reliable, Therefore, due to the fact that the isolation and amplification of nucleic acids are more accurate and sensitive than antigen-antibody reactions, the molecular techniques are considered a better alternative for these methods. Due to unavailability of immunohistochemical kits and its non-conventional usage for BRAF in Iran, it was not possible assess the results by this technique. Further studies, specially cytological studies in evaluating BRAF dimerization can confirm its role in disease. Studies show that the prediction of outcome in patients with colorectal cancer is considered as a complex clinical problem. In addition, despite the massive improvement in cancer cure, a large weakness can be seen in absence of definite treatment of patients which the use of multiple combined treatments is considered as an improving key. And also disease progression is associated with an increased risk of death. So the identification and use of new markers in diagnosis and utilizing them in treatment is a necessary and inevitable point and it can be concluded that, studies in this field have special value.

CONCLUSION

From the results, it can be summarized that BRAF gene expression is one of the diagnostic molecular markers in colorectal cancer and by using the paraffin-embedded block as a sample, different strategies can be applied in patients treatment, statistical researches, choosing the right decision by considering the proper drug and treatment. And finally it can has results inperforming the appropriate treatment strategy.

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