Original Article

Methylation pattern of ALX4 gene promoter as a potential biomarker for blood-based early detection of colorectal cancer

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Abstract

Background: To develop a non-invasive screening method for colorectal cancer, we evaluated the methylation of ALX4 gene promoter in serum samples from patients with colorectal cancer (CRC) and equal number of healthy individuals.

Materials and Methods: In serum samples from 25 patients with colorectal cancer and 25 healthy control subjects, isolated serum free-floating DNA was treated with sodium bisulfite and analyzed by methylation-specific polymerase chain reaction (MSP) with primers specific for methylated or unmethylated promoter CpG island sequences of the ALX4 gene.

Results: Methylation of the ALX4 gene promoter was present in the serum DNA of patients with adenoma and colorectal cancer. A sensitivity of 68% and specificity of 88% were achieved in the detection of promoter methylation in colorectal neoplasia samples. The difference in methylation status of the ALX4 promoter between the patients with colorectal neoplasia and the control group was statistically highly significant ($P < 0.001$).

Conclusions: The results indicate that this serum free DNA test of methylation of the ALX4 gene promoter is a sensitive and specific method. Therefore in combination with other useful markers it seems ALX4 has the potential of a clinically useful test for the early detection of colorectal cancer.

Key Words: Colorectal cancer, DNA methylation, Free-floating DNA, non-invasive colorectal cancer diagnosis

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INTRODUCTION

Currently, the incidence of colorectal cancer in Iranian older age subjects is very low compared to Western population, but the younger generation is experiencing an increased rate approaching the Western rates, and dramatically the burden of disease will increase in near future.[1-3] Environmental and genetic factors impress on the developing of CRC. Economic indices show that, since the 1979 Iranian revolution, living standards have significantly improved across country (statistical center of Iran, 2007, World Bank report 2006). Our country has experienced rapid development in socioeconomic status during the past three decade with significant

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lifestyle changes like sedentary lifestyle and the high fat and high protein diet, and low cereals and fibers typical of Western population.[3] Recently in Iran, epidemiologic studies[4,5] have indicated a rapid increase in the rate of CRC while there are still no preventive methods established. Together with the epidemiologic trend of CRC in Iran, new screening method is necessary in order to prevent morbidity and mortality for this lethal but preventable cancer in the country.[4] The number of new cases of CRC in Iran is 3641 each year, from which 2262 die of CRC annually, accounting for approximately 6.3% of all cancer deaths in Iran.[6] Early detection and resection of adenoma can significantly decrease the risk of mortality for patients with CRC.[7-9] CRC screening has been effective to reduce disease-specific mortality and several European countries practicing regular national screening program. The screening method almost entirely rely upon stool tests, with endoscopy employed as an adjunct in some countries.[10] The most widespread non-invasive screening methods currently used in Iran is faecal occult blood test (FOBT). This test although proved useful in CRC prevention but due to its low sensitivity and patient's compliance remained to be substituted by more sensitive with better compliance non-invasive tests.[11-15]

There are number of genes evaluated so far to examine their selective potentials to act as a desirable CRC blood-base screening biomarker in different populations.[16-18] Many of the human protein coding genes contain CpG islands in their promoters, hence abnormal cytosine methylation of these sequences result in inactivation of the prospective gene controlled by the promoter. There are numerous examples of tumor suppressor genes promoter methylation of CpG islands in various cancers, including colorectal cancer.[19] It is reported that the promoter methylation changes occur as early as precancerous lesions, such as adenomas, which indicates that the analysis of epigenetic DNA alterations may be useful for the early diagnosis of malignant diseases.[18,20] Interestingly it is evident that genetic and epigenetic alternations in serum free- floating DNA are identical to those found in primary human cancers.[21]

Based on the available data we choose to evaluate the ALX4 gene promoter methylation in subset of patients referred to GI wards of various hospitals of Isfahan located in the central part of Iran. ALX4 gene, homeodomain transcription factor ALX4, is located on the short (p) arm of the chromosome 11 at position 11.2. The ALX4 gene encoding a protein that is necessary for proper development throughout the body, especially in the skull and limb bones.[22,23] In various blood based studies using ALX4 gene promoter methylation, different levels of sensitivity and specificity were obtained, mostly with satisfactory achievement.[16,17] In this study, we assessed the methylation status of ALX4 gene in free floating plasma DNA samples from patients with CRC and healthy individuals, using methylation-specific polymerase chain reaction (MSP), as a potential marker for blood-based early detection and early screening of colorectal cancer.

MATERIALS AND METHODS

Study population

This study included equal number (25 + 25) of colorectal cancer patients and healthy individuals. The selection of patients was based on colonoscopy examination and pathology reports. Those who were diagnosed normal after colonoscopy examination constituted our control population. Our sample population solely confined to sporadic CRC, hence subjects with the familial history of CRC were excluded from our study. An informed consent was obtained from every subject prior to the study.

Sample collection

A total of 5 ml peripheral venous blood was collected from each participant. All blood samples were centrifugated at 3000 rpm for 10 min using a refrigerated centrifuge to separate sera. Then all sera samples were stored at –80 centigrade until use.

DNA extraction

Free-circulating DNA extracted from 200 μl of serum with QIAmp blood mini kit (Qiagen, Germany) according to the manufacture's protocols and stored at –80 centigrade until use.

Bisulfite conversion

Bisulfite treatment of DNA samples performed using EpiTect Bisulfite Kit (Qiagen) which converts only unmethylated cytosines to uracils, according to the manufacturer instructing manual.

MSP

3 μl of bisulfite converted DNA was used as template for MSP. Briefly, the reaction performed in a 25 μL volume consisting of 2.5 μL 10X PCR buffer, 200 μM dNTP mixture, 10 pM of each forward and reverse primers [Table 1], and 2.5 U hot start TaKaRa Taq polymerase. The thermal cycling applied as follows: 95°C for 15 min, followed by 40 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 5 min. DNA extracted from the peripheral blood normal lymphocytes was included

Table 1: Sequences of the primer used in MSP

Primer	Sequences (5′-3′)	Annealing temperature	Product size (bp)
MF for ALX4 gene	5′GGGTAAGGAGTGATA AGTATAAGTTTTTTC 3′	58.0 C°	118
MR for ALX4 gene	5′CATACCTAACTTACGC AAACGAC 3′	57.9 C°	118
UF for ALX4 gene	5′GGGTAAGGAGTGATA AGTATAAGTTTTTTT 3′	57.7 C°	125
UR for ALX4 gene	5′CAACATTCATACCTA ACTTACACAAACA 3′	59.7 C°	125

as unmethylated control and placental DNA was treated *in vitro* with Sss I methyltransferase (New England Biolabs, Beverly, MA) as a positive control for methylated alleles.

Statistical analysis

Statistical analysis was performed using Fisher exact test (2 sided) and SPSS v.16, *P* value less than 0.05 was considered significant.

RESULTS

Methylation of ALX4 gene promoter in serum of patients with colorectal cancer

We assessed methylation status of ALX4 gene promoter in serum DNA from the patients and control groups by MSP reaction. Methylation of ALX4 was detected in 17 of 25 CRC patients [Figure 1] and 3 out of 25 controls. Patients had mean age of 67 years. 44% of cases were female and 56% of patients were male. Therefore, analysis of ALX4 methylation in peripheral blood serum revealed a sensitivity of 88% and specificity of 68%, respectively. The present study shows that methylation levels were significantly higher in CRC patients compared to their normal counterparts ($P < 0.001$).

DISCUSSION

One of the main problems existing with present CRC screening methods is low patients compliance. CRC has all features which make it a good candidate for mass screening. These criteria including high incidence and prevalence, high mortality in advanced disease, easily curable provided to be diagnosed in its early stages. CRC screening tests can be divided into direct and indirect categories.[24] Stool-based tests (guaiac Faecal Occult Blood Test, gFOBT; Faecal Immunochemical Test, FIT; Faecal DNA tests) diagnose cancers and large adenomas indirectly through detecting their by-products (blood, DNA) in the stool. The main drawback of indirect tests is that they favor cancer detection rather than adenomas, and so provide less opportunity to impact on cancer incidence. Direct tests such as flexible sigmoidoscopy,

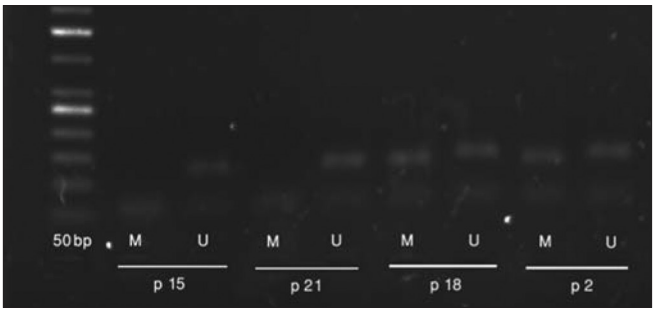

Figure 1: ALX4 Methylation Status in affected CRC patients using methylated and unmethylated primers on 2.5% agarose gel stained with EtBr

colonoscopy, Computed tomographic colonography (CTC), directly visualize the target lesion, either in cancers or adenomas stage. Such tests can impact on both cancer mortality and incidence.[25,26] Compared with indirect tests, direct tests are more invasive and costlier. Also it is reported that the gFOBT and FIT tests have per patient sensitivity of 11-20 and 27-48% respectively for advanced neoplasia and of 13-38 and 56-88% for CRC.[27-31] Sigmoidoscopy has a sensitivity of approximately 83% for advanced neoplasia and 58-75% for CRC.[29,32] Colonoscopy has a sensitivity of 88% for all advanced neoplasia, of 98% for advanced neoplasia ≥10 mm, and of 95-97% for CRC.[29,33,34]

Although colonoscopy is a gold standard protocol for CRC screening and detection but the main disadvantage of this procedure is its very low patient's compliance and high cost. On average less than 30% of the eligible population in the US and Europe are willing to undergo this procedure for screening. Various studies have shown that there is a high level of circulating DNA in peripheral blood of patients with malignancies, and it is found that circulating DNA can represent a variety of tumor characteristic changes, such as mutation of oncogenes and tumor suppressor genes, microsatellite instability, chromosome translocation and gene expression changes. Presence of elevated DNA in the serum which is proved to be derived from tumor promoted the search for amplifiable tumor DNA markers in patient blood.[15-17] The developments of blood-based cancer-detection tests can be improve patient compliance and thereby increase the detection of disease at earlier stages.[18,19] In a study performed by Tanzer *et al*. they obtained a sensitivity level of 83.3% and specificity of 70% for ALX4 gene promoter methylation in plasma sample of mostly colorectal precancerous lesions. So this level of sensitivity is almost comparable with that of invasive colonoscopy. On a validation study we analysed ALX4 promoter methylation in serum samples of 25 CRC patients and their normal control counterparts. High sensitivity of

88% obtained which is higher than that of previously reported for this gene.[16] This level of sensitivity is well compared with the gold standard procedure, colonoscopy. Being a non-invasive method performed for the first time in Iran with satisfactory sensitivity level make this strategy as a valuable breakthrough in colorectal cancer screening. We hope that future further characterization of this blood based procedure with more diverse DNA markers previously studied by our group,[35-37] make it an influential non-invasive method for CRC screening in Iran.

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