

A Study on Tumor Suppressor Genes Mutations Associated with Different Pathological Colorectal Lesions.

Thesis

**Submitted for partial fulfillment of the requirements for
the Ph.D. degree of Science in
Biochemistry**

By

Salwa Naeim Abd El-Kader Mater
M.Sc. in Biochemistry, 2002

*Biological Applications Department
Nuclear Research Center
Atomic Energy Authority*

Under the supervision of

Prof. Dr. Amani F.H Nour El-Deen

**Professor of Biochemistry
Biochemistry Department
Faculty of Science
Ain Shams University**

**Prof. Dr. Abdel Hady Ali
Abdel Wahab**

**Professor of Biochemistry
and Molecular Biology
Cancer Biology Department
National Cancer Institute
Cairo University**

Prof. Dr. Mohsen Ismail Mohamed

**Professor of Clinical Pathology Biological
Applications Department
Nuclear Research Center
Atomic Energy Authority**

Dr. Azza Salah Helmy

**Assistant Professor of Biochemistry
Biochemistry Department
Faculty of Science
Ain Shams University**

2011

دراسة على الطفرات في الجينات المثبطة للأورام المصاحبة لإصابات القولون و المستقيم.

جزء متمم للحصول على درجة الدكتوراه
قسم الكيمياء الحيوية - كلية العلوم
جامعة عين شمس

رسالة مقدمة من

سلوى نعيم عبد القادر مطر
ماجستير العلوم في الكيمياء الحيوية (2002 م)

تحت إشراف

أ.د /عبدالهادي على عبدالوهاب	أ.د/ أماني فاروق حسين نور الدين
أستاذ الكيمياء الحيوية والبيولوجيا الجزيئية	أستاذ الكيمياء الحيوية
قسم بيولوجيا الأورام	قسم الكيمياء الحيوية
معهد الأورام القومي - جامعة القاهرة	كلية العلوم - جامعة عين شمس

د / عزة صلاح حلمي	أ.د/ محسن إسماعيل محمد
أستاذ مساعد الكيمياء الحيوية	أستاذ الباثولوجيا الإكلينيكية
قسم الكيمياء الحيوية	شعبة تطبيقات النظائر المشعة
كلية العلوم - جامعة عين شمس	مركز البحوث النووية - هيئة الطاقة الذرية

قسم الكيمياء الحيوية
كلية العلوم
جامعة عين شمس

2011

Abstract

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western world. In Egypt; there is an increasing incidence of the disease, especially among patients ≤ 40 years age. While CRC have been reported in low incidence rate in developing countries, it is the third most common tumor in male and the fifth common tumor in females in Egypt. Early diagnosis and surgical interference guarantee long survival of most CRC patients. Early diagnosis is impeded by the disease onset at young age and imprecise symptoms at the initial stages of the disease. As in most solid tumors, the malignant transformation of colonic epithelial cells is to arise through a multistep process during which they acquire genetic changes involving the activation of proto-oncogenes and the loss of tumor suppressor genes. Recently, a candidate tumor suppressor gene, KLF6, which is mapped to chromosome 10p, was found to be frequently mutated in a number of cancers. There are some evidences suggesting that the disruption of the functional activity of KLF6 gene products may be one of the early events in tumorigenesis of the colon. The main objective of the present study was to detect mutational changes of KLF6 tumor suppressor gene and to study the loss of heterozygosity (LOH) markers at chromosome 10p15 (KLF6 locus) in colorectal lesions and colorectal cancer in Egyptian patients. The patients included in this study were 83 presented with different indications for colonoscopic examination. Selecting patients with colorectal pre-cancerous lesions or colorectal cancer was done according to the results of tissue biopsy from lesion and adjacent normal. The patients were classified into three main groups; (GI) Cancerous group, (GII) polyps group including patients with adenomatous polyps (AP), familial adenomatous polyps (FAP) and hyperplastic polyps (HP) and (GIII) Inflammatory Bowel Diseases (IBD) including patients with ulcerative colitis (UC) and Crohn's disease (CD). Purified DNAs which were extracted from the tissue samples, PCR amplified and subjected to the following examinations: **1-** Detection of KLF6 mutations by using SSCP-silver staining technique and DNA sequencing by using BigDye Terminator v3.1 Cycle Sequencing kit using Biosystem automated sequencer (The ABI PRISM 3100 Genetic Analyzer). **2-** Determination of Loss of heterozygosity (LOH) on chromosome 10p15 regions (KLF6-locus) by using three microsatellite markers which includes KLFM1, KLFM2, and KLFM4. Data from the present study could be

summarized as follows: In GI, 55.3% of cases had abnormalities in KLF6 gene (mutations and LOH). LOH was detected in 29% of investigated samples while KLF6 mutations were detected in 44% of cases. In GII, 57% of cases had abnormalities in KLF6 gene (mutations and LOH). LOH was detected in 55% of investigated samples while mutations of KLF6 gene were detected in 26% of investigated samples. In GIII, 50% of samples had abnormalities in KLF6 gene (mutations and LOH). LOH was detected in 36.4% of investigated samples while mutations of KLF6 gene were detected in 27.3% of investigated samples. Most of the mutations reported were of the missense and /or Transversion type and were almost in exon 2. In conclusion, our data highlight for the first time a role of KLF6 gene in the progression of Egyptian colorectal carcinogenesis where the results suggest that KLF6 gene alteration is involved in the progression of Egyptian colorectal carcinogenesis from both sporadic adenomatous polyps and ulcerative colitis pathways. Detecting mutational sites differing from that detected in western populations may be a characteristic of Egyptian CRC due to environmental and genetic factors. The detections of such genetic abnormalities may also be used as a marker for the early uncovering of colon cancer cases. It is recommended that those who have pre-neoplastic colon lesions in which the KLF6 gene has mutated or lost its heterozygosity should experience more frequent colonoscopic examinations for the detection of doubtful malignant changes. The present study also paves the way to further research needed to elucidate the possible role of KLF6 protein in the transactivation of other genes involved in cell cycle regulations and apoptosis. It also supports the possibility of using KLF6 gene as a target for gene therapy in colorectal cancer.

List of Abbreviations

ACF	aberrant crypt foci
AFP	Alpha Fetoprotein
AP	adenomatous polyps
APC	Adenomatous polyposis coli gene
APS	Ammonium persulfate
bp	Base pair
BTEB1	basic transcription factor element binding protein
BTEB2	basic transcription factor element binding protein 2
CD	Crohn's disease
CEA	Carcinoembryonic Antigen
CIN	Chromosomal instability
COPEB	core promoter element-binding protein
CPBP	core promoter binding protein
CPBP	Core Promoter Binding Protein
CPE	core promoter element
CRC	Colorectal cancer
DCBE	Double contrast barium enema
DCC	Deleted in colorectal cancer gene
ddH₂O	Deionized distilled water
DNA	Deoxy-ribonucleic acid
DRE	Digital rectal exam
EDTA	Ethylene Diamin tetraacetate
ER	Estrogene receptor
FAP	familial adenomatous polyps
FKLF	fetal erythroid Kruppel-like factor
FOBT	Fecal occult blood test
Ha-MSV	Harvey murine sarcoma viruses
IBD	Inflammatory Bowel Disease
IRB	institutional review board
Kd	Killo Dalton
Ki-MSV	Kirsten murine sarcoma viruses
KLF6	Krüppel-like factor 6
KLF_family	Krüppel-like transcriptional factors family
Lis-SSCP-	low ionic strength- Single-strand conformation
loading buffer	polymorphism- loading buffer
LOH	Loss of heterozygosity
MMR	DNA mismatch repair
MSI	micro satellite instability
OD	Optical density

PCR-LIS-	polymerase chain reaction-low ionic strength-
SSCP-silver	Single-strand conformation polymorphism- silver
staining	staining method
PSA	Prostate Specific Antigen
PSG	Pregnancy-Specific Glycoprotein genes
PSG	pregnancy-specific glycoprotein
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism analysis
TAE	Tris-Acetate-EDTA buffer
TBE	Tris-Borate-EDTA buffer
TE buffer	Tris EDTA buffer
TEMED	N,N,N',N'-tetramethylene diamine
TGF β	transforming growth factors β
U.C	ulcerative colitis
UC-CRC	Ulcerative colitis- colorectal cancer pathway
pathway	
UKLF	ubiquitous Kruppel-like factor
UTR	Un-translated regions
uPA	urokinase type plasminogen activator
10p15	Short arm of chromosome 10 (locus 15)

List of Tables

<i>TABLE NO.</i>	<i>TITLE</i>	<i>PAGE</i>
1-	The clinical data for the patients of adenocarcinoma group (I).....	48
2-	The clinical data for the patients of polyps group (II).....	49
3-	The clinical data for the patients of IBD group (III).....	50
4-	Primer sequences and the size range for klf6 exons used.....	57
5-	PCR conditions for KLF6 gene (exons 1-4) amplifications.....	58
6-	The primer sequences and the size of the amplified fragments for the KLFM1, KLFM2 and KLFM4 markers.....	67
7-	PCR conditions for KLF6 gene (LOH markers) amplifications.....	68
8-	The pattern of mutations, codon no. and amino acid changes in the examined colon cancer cases (GI).	82
9-	The pattern of mutations, codon no. and amino acid changes in the examined colon polyp cases (GII).....	85
10-	The pattern of mutations, codon no. and amino acid changes in the examined colon IBD cases (GIII).....	88
11-	The clinicopathological data of some cases in GI.....	104

12-	Statistical relations in GI.....	104
13-	The clinicopathological data of some cases in GII.....	105
14-	The clinicopathological data of some cases in GIII.....	107

List of Figures

<i>FIGURE NO.</i>	<i>TITLE</i>	<i>PAGE</i>
1-	Colon anatomy.....	12
2-	Diagram shows the progress of Colon cancer start from colon polyps.....	16
3-	Colorectal anatomy diagram show different lesions and cancer anatomy.....	17
4-	Schematic diagram for different colorectal cancer stages.....	19
5-	Proposed adenoma to carcinoma sequence in colorectal cancer... ..	29
6-	Schematic representation of the putative steps in colorectal cancer progression.....	36
7-	KLF6 protein structure.....	41
8-	Functional domains of KLF6 protein.....	42
9-	Location of KLF6 gene on chromosome 10.	43
10-	Genomic structure of KLF6 gene.....	44
11-	A schematic representation of the SSCP technique.....	56
12-	Genomic DNA isolated from tissue biopsies for cases no. 1-8 of the CRC group.	72
13-	The percentage distribution for KLF6 mutation in the three different studied groups.....	73

14-	The percentage distribution for mutations in the two age categories in the three examined groups.....	74
15-	The percentage distribution for mutations in the males and females in the three examined groups.....	75
16-	The percentage distribution for mutations of KLF6 gene in different lesion sites in the three examined groups.....	76
17-	The percentage distribution for mutations of KLF6 gene in different pathological grades of colon cancer cases in cancer group.	77
18-	The percentage distribution for mutations of KLF6 gene in the two categories of ulcerative colitis cases.....	78
19-	The percentage distribution for mutations of KLF6 gene in the two size categories of polyps group.	79
20-A	The percentage distribution for mutations of different exons of KLF6 gene in cancer group (GI).	80
20-B	The percent of mutations for KLF6 gene exons according to sequencing results in cancer group.	81
21-A - B	Detection of KLF6 gene mutations in CRC sample no. 1. (A)- SSCP analysis for exon 2 for CRC samples (T1-T5)..... (B)- Direct sequence analysis representative sample (case no. 1) of CRC for both normal and tumor tissues.....	83 83

22-A	The percentage distribution for mutations of different exons of KLF6 gene in Polyps group (GII).....	84
22-B	The percentage of mutations for KLF6 gene exons according to sequencing results in polyps group.....	85
23-	SSCP-silver staining analysis for exon 2 of KLF6 gene for polyps tissues.	86
24-A	The percentage distribution for mutations of different exons of KLF6 gene in IBD group.....	87
24-B	The percentage of mutations for KLF6 gene exons according to sequencing results in IBD group.....	88
25-	SSCP-silver staining analysis for exon 1 of KLF6 gene for IBD tissues.....	89
26- (A,B&C)	PCR product for the 3 markers used in the present study (KLFM1, KLFM2 and KLFM4) without using radioactive ³² P in the reaction.....	91
27-	Percentage of LOH in cancerous group for the 3 different microsatellite markers used...	92
28-	LOH study in cancerous group for KLFM1 marker.....	92
29-	Individual distribution of LOH analysis in cancer group for the 3 microsatellite markers examined.....	93
30-	The percentage distributions of LOH markers with different clinicopathological examined factors.....	94

31-	Percentage of LOH in IBD group for the 3 different microsatellite markers used.....	95
32-	LOH study in IBD group for KLFM4 and KLFM2 markers.	96
33-	Individual distribution of LOH analysis in IBD group for the 3 microsatellite markers examined.....	97
34-	The percentage distributions of LOH markers with different clinicopathological examined factors.....	98
35-	Percentage of LOH in polyps group for the 3 different microsatellite markers used.....	99
36-	LOH study in polyps group for KLFM4 and KLFM2 markers.	100
37-	Individual distribution of LOH analysis in polyps group for the 3 microsatellite markers examined.	101
38-	The percentage distributions of LOH markers with different clinicopathological examined factors.....	102
39-	The distribution of KLF6 status results in GI	103
40-	The distribution of KLF6 status results in GII..	105
41-	The distribution of KLF6 status results in GIII.....	106

Contents

Abstract.....	i
Introduction.....	1
Aim of the work.....	6
Review of Literature.....	7
I- An overview on cancer.....	7
• Cancer definition	7
• Benign and malignant tumors	7
• Causes of Cancer.....	9
II- COLORECTAL LESSIONS AND CANCER.....	11
(1) Anatomy of the colon	11
(2) Colorectal lesions.....	13
(3) Colorectal cancer.....	17
(a)-Colorectal cancer progress	18
(b)-The symptoms of colorectal cancer.....	20
(c) -Risk factors of colorectal cancer.....	20
(d)- Methods of colorectal cancer screening.....	21
(4) Epidemiology of colorectal lesions and cancer.....	25
(5) Colorectal carcinoma in Egyptian patients.....	27
(6)- Molecular pathways of colorectal cancer.....	28

III- Gene involved in the pathways of colorectal cancer.....	30
IV- KLF6 tumor suppressor gene.....	37
(1)- Introduction.....	37
(2)- KLF6 protein.....	40
(3)- KLF6 gene nomenclature and identification.....	43
(4)- KLF6 tumor suppressor gene and Cancer.....	45
Subjects and Methods	47
Results	72
Discussion	108
Summary	120
References	123
Arabic summary	

INTRODUCTION AND AIM OF THE WORK

Introduction:

Normal development is a balance process, which includes proliferation and cell death. Indeed both proliferation and apoptotic cell death are very complex process that involves the participation of many genes. In both events, the tumor suppressor genes are the most important and studied genes (*Jung & Messingm, 2000*). The carcinogenic procedure is a multistep process involving several genetic alterations that eventually ends in malignant transformation. The study of carcinogenic procedure is very important, because it does not only shed light on some critical steps in the progress of carcinogenesis but may also provide a rational approach for early diagnosis of cancer (*Mendoza-Rodriguez & Cerbon, 1995*).

Although some diagnostic markers are available that are assayable from blood or tissue samples, e. g. Carcinoembryonic Antigen (CEA), Alpha Fetoprotein (AFP) or Prostate Specific Antigen (PSA), the assays using these markers have not, to date, been markedly predictive of the presence of cancer in these individuals, as verified by other clinical diagnoses. The sensitivity and specificity of these assays has been disappointingly low. Time-consuming and labor-intensive clinical assessments (e. g. palpations, x-rays, mammograms, biopsies) have remained the accepted methods for diagnosing cancer. Thus, a need exists for a biomarker that is predictive of the presence of cancer or of an increased risk of developing a cancer in the individual. In particular, a need exists for a marker and an assay to measure the presence and amount of this marker for individuals with an early stage of cancer. If such diagnostic test is available, early treatment with beneficial outcomes would be more likely than at present (*Srivastava and Gopal-Srivastava, 2002*).

One of the most important discoveries in cancer biology was the cancer arise as a result of cumulative genetic changes in cells. The progression of a tumor from normal cells to pre- cancerous ones, to cancer and then to local invasion and finally metastasis is the result of the clonal expansion of cells that have acquired a selective growth advantage, which allows them to outnumber neighboring cells. This