Study of Human Cytomegalovirus nucleic acid in specimens of colorectal adenocarcinoma and villous adenoma using Polymerase chain reaction (PCR) technique

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الخلاصة

على الرغم من التقدم المط !ارد في فهم المسالك الجزيئية لمسببات السرطان البشري والتي تسبب تغيير تلك المسالك إلا إنها لازالت غير معروفة.

لوحظ بان الفيروس المضخم للخلية البشرية يسبب استحالة خلايا الوسط ألزرعي ، مع ذلك، فان الحامض النووي للفيروس لم يتم إيجاده في أكثر الخلايا المتحولة ،كذلك فان الطريقة التي يكون بها هذا الفيروس مسرطنا لازالت مبهمة.

لمزيد من التحقيق في هل أن الفيروس HCMV له علاقة في إحداث سرطان القولون والمستقيم ،أخذنا 13 عينة لسرطان القولون والمستقيم ،و 5عينات للورم ألغدي الزغابي للفحص وتقنية سلسلة تفاعل أنزيم البلمره(PCR) .

نتائج (PCR) ، لوحظ وجود نتائج ايجابية على شكل حزمة مفردة نوعية في 9 عينات من أصل 13 عينة لسرطان القولون والمستقيم بنسبة (69,23 %) ، وفي 3 عينات من مجموع 5 عينات للورم ألغدي الزغابي بنسبة (60 %) ، وذلك باستخدام البادئ المعيّن للسلسلة الجينية للجين المبكر (PP65) والذي وجد في الحامض النووي البشري .

إن وجود المادة الوراثية لفيروس HCMV في السرطان البشري هو نقطة ساخنة لان هذا الفيروس يصيب الأعضاء بشكل عالي وكامن للأفراد الطبيعيين ،وكذلك يحتمل أن الفيروس يغير الخاصية البيولوجية للخلايا السرطانية بدون أن يتدخل في إحداث السرطان.

Abstract

Although the rapid advance in the understanding of molecular pathways underlying human tumorigenesis causes that initiate dysregulation of the pathways remain largely unknown. Human cytomegalovirus has been shown to transform cultured cells, however, viral DNA is not detected in most transforming cells, and the mechanism by which HCMV might contribute to oncogenesis has remained obscure.

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For further investigation of HCMV role in human colorectal tumorigenesis, we have tested 13 specimens of colorectal adenocarcinoma & 5 specimens of villous adenoma using polymerase chain reaction (PCR) techniques.

The results of PCR assay showed specific single band in 9 of 13 specimens (69.23%) of colorectal adenocarcinoma, and 3 of 5 (60%) of villous adenoma, by using specific primer targeted directly to the early gene (pp65) that expressed within host DNA, the results read by agarose gel electrophoresis & UV – transilluminator.

The presence of HCMV genetic information in human tumors is a hot point because HCMV can infect the organs latently in a high percentage of normal individuals, it also remains possible that HCMV modulates the biologic properties of malignant cells without being directly involved in carcinogenesis.

Introduction

The world-wide incidence of colorectal carcinoma varies dramatically, from 3.4 cases per 100.000 population in Nigeria to 35.8 cases per 100.000 population in the rest countries (Schottenfold & Winawer, 1982). Adenocarcinoma of the large bowel is the third leading cause of cancer death in the U.S. .The over all incidence of colorectal cancer in the U.S. is nearly identical in men and women, with the mean age of presentation between 60 to 65 years of age (Landis *et al.*, 1998).

In Iraq, according to the Iraqi cancer registry reports, colorectal carcinoma is one of the commonest ten cancers by site and gender among Iraqi patients. It is representing about 55.2 % among males, 44.7 % among females and, 4.75% among other cancers (Iraqi cancer Board, 2001).

Few viruses species have been detected in human cancers , and in some human tumors, these viruses probably play a critical role in carcinogenesis since they are present early during the process of cancer development and are constantly detectable in the tumor cells, such as Epstein – Barr virus (Macsween & Crawford , 2003), human herpes virus – 8 (HHV-8) (Ganem,1997), and human

papillomavirus (HPV) (Zur H., 2000),other viruses have been incriminated in human carcinogenesis but there is still a hard debate regarding their direct implication in cancer for example human cytomegalovirus (Cobbs *et al.*, 2002).

Recently, it was hypothesized that HCMV might be associated with colorectal carcinoma progression; numerous studies had linked HCMV infection with colorectal carcinoma.

Materials & Methods
Polymerase chain reaction (PCR)
Materials:

- DEXPAT DNA Extraction Reagent (TAKARA MIRUS
 BIO INC., no.9091, 100 reactions) from paraffin-embedded tissue.
- Cytomegalovirus PCR detection kit (50 reaction) (cat. no.PR 7836c-cinnagen Inc.).
- Sterile pipettes (10 µl and 20 µl).
- Sterile eppendroff tubes.
- High speed centrifuge (Hettich centrifuge, Germany).
- Shaker (Vortex mixer).
- Microfuge (Minispin eppendrof microfuge).
- Thermal cycler (PXE 0.2).
- Hood class II (Advanced bio safety cabinet class II).
- High melting agarose gel.
- Gel electrophoresis.
- UV-transilluminator.
- Oven.
- Plastic gloves.
- Marker (snowman marker, USA).

Methods
Eighteen paraffin blocks (13 of colorectal adenecarcinoma & 5 of villous adenoma) were selected for PCR from cases which appeared positive HCMV early protein by IHC assay.

The surface of the microtome and the area of the laboratory bench were wiped with cloths socket in 100 % ethanol to remove adherent flaks of wax .Each histological block was wiped with 100 % ethanol , inserted into the microtome carriage , trimmed and the waste was carefully discarded . The microtome blade and the operator's gloves were changed. The steps of DNA extraction according to the commercial kit (TAKARA MIRUS BIO INC) as the following:

• Cutting 1-3 tissue sections, (10 µm paraffin -embedded tissue sections), and adding the sections into the sterile eppendrof tube by sterile disposable stick.

• Added 20 drops of DEXPAT DNA extraction reagent (after shaking) into eppendroff tube that contain tissue sections, and then shaking gently.

• Incubate the eppendroff tube at 100 ºC for 10 minutes in the oven to dissolve the wax from the paraffin sections.

• Centrifuge the eppendroff tubes by using high speed centrifuge at 12.000 rpm for 10 minutes at 4ºC.

• The result seen as 3 layers (paraffin thin layer, supernatant containing DNA and resin respectively).

• Remove the paraffin thin layer to the tube wall and collect the supernatant using micropipette without tissue debris.

• Take 5µl of the supernatant as the template for PCR reaction.

PCR amplification protocol
The procedure was applies according to the leaflet of the commercial kit (Cinnagen, Iran).

• Take out the kit and unfreeze the tubes.

• Label 0.5 ml tubes for amplification reactions (8 tubes for each run involved positive and negative).

• Added the following reagents for each tube, 1x PCR mix 20µl, and Taq DNA polymerase 0.3µl ,because there is difficult to calculate 0.3µl, we prepared these reagents together for each run (8 tubes) and added 20.3 µl (1x PCR mix plus Taq DNA polymerase) to each tube.

- To avoid contamination all reagents must be taken with separate clean tips.
- Mix the mixture thoroughly by shaking and spin.
- To each tube, 20μl were added (one drop) mineral oil.
- 5 µl of DNA specimen was added by specific pipette.
- Close tubes; spin the mixtures on microfuge for 3 seconds at 8000 rpm.
- Transfer the tubes to preheated thermocycler and start the following program:-

Stage one (one cycle)

_____
Step 1 (95ºC-180sec.)
Step 2 (62ºC- 40sec.)
Step 3 (72ºC-40sec.)
Stage tow (45 cycles)

_____
Step 1 (93ºC-40sec.)
Step 2 (61ºC-40sec.)
Step 3 (72ºC-40sec.)

Gel Electrophoresis
The PCR products were identified using agarose gel electrophoresis followed by the detection of the DNA bands:
Materials
- Agarose 2 %(Promiga, USA).
- Gel chamber.
- 1x TAE (Tris-acetate-EDTA Buffer).
- Ethidium bromide solution (5 µl/100ml).
- Electrical heater.
- Adjustable pipette.
- Digital camera (Sony, Japan).
- UV –transilluminator (approx. 200-300 nm, consort- Germany).
- Power supply (Consort-Germany).
- Deionized distilled water.

Methods

- Tow percentage of high melting agarose was prepared by boiling 0.8 g of agarose in 40 ml of 1x TAE until the solution becomes clear .

- The solution was allowed to cool, and then 2 µl of ethidium bromide solution was added, then agarose solution poured into topped plate to make gel, and put specific-dividing cast on the gel to make pockets.

- After the polymerization (about 3 hours ,at room temperature), the gel was placed into the gel chamber that was filled with 1x TAE buffer, the gel pockets should be completely covered with buffer.

- 10 µl from PCR- mixture tubes applied into each gel pocket.

- The electrophoresis was then carried out for 10 minutes at 100 V/ cm (distance of electrodes).

- When the electrophoresis was completed, the gel was placed on a UV-transilluminator by suitable face protection against UV radiation should be worn. A digital picture was made for evaluation and documentation of the results.

- For evaluation, the pattern of a specific single band was compared with a positive and negative control.

Results
Expression of HCMV nucleic acids

Tissue sections were collected from (13) case of colorectal adenocarcinoma and (5) cases of villous adenoma, DNA was extracted from each case using TakaRa DEXPAT from paraffin – embedded tissues.

DNA extracts were tested to present the early gene (pp65) using specific primer from (Cinnagen company .Iran).

The amplified DNA was determined using agarose gel electrophoresis. Successful amplification resulted from the generation of DNA fragment of defined length (222 kbp). In 9 of 13 cases (69.23%) colorectal adenocarcinoma and 3 of 5 cases(60%) villous adenoma appeared positive inform of single band at the same level of the positive control , the negative control appeared negative clearly (Table 1&2) (Figure 1) .

Table 1. The cases were appeared positive with PCR assay.

Cases no.	Histopathological diagnosis *	Stage	Grade
Positive cases			
1	Adenocarcinoma	B2	G1
2	Adenocarcinoma	B2	G11
3	Adenocarcinoma	C1	G11
4	Adenocarcinoma	B2	G11
5	Villous adenoma		
6	Villous adenoma		
7	Adenocarcinoma	B2	G11
8	Adenocarcinoma	C1	G11
9	Adenocarcinoma	B2	G11
10	Adenocarcinoma	B1	G111
11	Adenocarcinoma	C1	G11
12	Villous adenoma		

Table 2.The cases were appeared negative with PCR assay

Cases no.	Histopathological diagnosis *	Stage	Grade
Negative cases			
1	Villous adenoma		
2	Villous adenoma		
3	Adenocarcinoma	C1	G11
4	Adenocarcinoma	B2	G11
5	Adenocarcinoma	B2	G11
6	Adenocarcinoma	B1	G1

*The diagnosis, stage, and grade according to the patients' reports in the private lab.

A   B

(Figure 1) PCR analysis of early protein (pp65) in colorectal adenocarcinoma and villous adenoma ,early protein was amplified from human DNA that extracted from paraffin-embedded tissues as a template , electrophoresis was carried out in 2% agarose of 100 v/cm for 15 minutes and stained with ethidium bromide .

A= adenocarcinoma , V.A.= villous adenoma , N= negative control, P= positive control. As the following from left to right (A&B respectively):

Discussion

The results of PCR assay showed a specific single band of early protein in 9 of 13 specimens (69.23%) of colorectal adenocarcinoma and 3 of 5 specimens (60%) of villous adenoma as shown in (Table 1).

The detection of HCMV DNA in tumor tissues isolated from patients biopsies ,imply a relationship between HCMV and several cancers including cervical carcinoma ,prostate cancer , and adenocarcinoma of the colon (Shen *et al* ,1997;Doniger *et al* ,1999). Our study would explain the presence of HCMV nucleic acids within the colorectal adenocarcinoma and villous adenoma, although these data don't establish a causal role for HCMV in colorectal pathogenesis ,a wealth of existing data indicates that HCMV could facilitate colorectal adenocarcinoma progression. A point should be noted here that ,The progression through the cell cycle is regulated by cyclins and their associated cyclin-dependant kinases (cdks) which are only active when complexes with their particular cyclin partner (Pines ,1993 ; Sherr,1993; Cordon-Cardo ,1995),and progression through the cell cycle occurs as a result of phosphorylation by cdks of specific substrate molecules such as the retinoblastoma –susceptibility protein Rb (Sinclair *et al* ,2000) ,

other regulatory steps are also involved ,these include activation of the cyclin – cdk complex by phosphorylation and dephosphorylation of specific sites on cdks or inhibition by interaction with so called cdks inhibitors such as p21,p61 and p27(Pines,1993; Sherr,1993;Cordon-Cordo ,1995).Generally ,high levels of cdk activity are indicative of cell cycle progression ,while terminally differentiated or quiescent cells have low levels of cdk activity and elevated levels of mitotic inhibitions(Sinclair *et al* ,2000).

The p53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that transactivates the promoters of many p53-responsive target genes including the cyclin-dependant kinase inhibitors ,p21 which mediate cellular growth inhibition ,p53 induction can also trigger signals leading to cell death or apoptosis (El-Deiry *et al* ,1993;Harper *et al* ,1993), Zhu showed that HCMV infection of human cells block the induction of apoptosis (Zhu *et al* ,1995).

Chen and colleagues investigat the mechanism responsible for the decrease of p21 levels after HCMV infection by measuring p21 RNA and protein levels in permissive human lung (LU) ,by using western blot analysis ,they showed ,p21 levels declined sharply over the next 24 hours and remained at very low level through 96 hours post infection (Chen *et al* ,2001), on the other hand , Cinalt attempted to investigate whether HCMV infection is associated with reduced TSP-1 production ,he found in conjunction with accumulation p53 that TSP-1 mRNA and protein expression were significantly reduced in HCMV –infection cultured human fibroblast (Cinalt, 1999).

RB is known to be hypophosphorylated in G0 and early G1 phase of the cell cycle and progression through G1 into S phase is associated with increased RB phosphorylation (Buchkovih *et al* ,1989 ;Chen ,1989 ;Decaprio ,1992) ,hypophosphorylated RB protein induces cell cycle arrest (Goodrich *et al* 1991 ;Hinds *et al* ,1992),that's to say, active RB (unphosphorylation form) proteins repress the expression of E2F- target genes by binding to E2F proteins at their C-terminal transactivation domain and recruiting

transcriptional repressors to promoters of target genes (Harbour & Dean ,2000)and Inactivate RB (phosphorylation form) proteins by cyclin /cyclin –dependent kinase complexes activated during mid-late G1 ,the RB proteins no longer bind E2F proteins (Nevins, 1998),The E2F proteins play an essential role in regulating the expression of genes required for DNA replication (Trimarchi & Lees , 2000). On the other hand, the infection of T2 cells (human embryonic carcinoma cell line) results in an increase in the hyperphosphorylated form of RB as early as 24 hours post infection (Sinclair, 2000).

According to our results, we conclude, successful extraction of HCMV genome from cellular DNA of cancerous tissue, also,

The presence of HCMV proteins in the colorectal biopsies opens a way for advanced investigations about the oncogenic role of this virus, in addition to that, it makes certain protection to avoid the cancer occurrence by treatment of HCMV infection or test from time to time to checking the colon and rectum lumen from any foreign mass.

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