Detection of Human Cytomegalovirus Pp65 in colorectal adeno-carcinoma and villous adenoma using Immunohistochemistry (IHC) technique

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Abstract

Colorectal adenocarcinoma is the third most common cancer in men and women and is the second leading causes cancer death. Recently detected Human Cytomegalovirus (HCMV) proteins in a high proportion of human colorectal tumors. These finding raise the chance of whether persistent HCMV infection can induce oncogenic pathways that eventuate in colorectal adenocarcinoma.

This study investigates whether HCMV participates in human colorectal tumorigenesis by the detection of HCMV Pp65 within epithelial cells of colorectal carcinoma using Immunohistochemistry (IHC). We obtained formalin–fixed, paraffin – embedded specimens of adenocarcinoma, villous adenoma, and normal tissues from the margins of the excision as a control. In this study, 55 specimens were classified into three groups: Adenocarcinoma, Villous adenoma, and control group, all groups have been tested by IHC to detect the presence of HCMV proteins using mouse monoclonal antibodies to an early protein (pp65). The results of IHC assay showed specific nuclear and cytoplasmic reaction of HCMV proteins within the epithelial cells of colorectal adenocarcinoma (75.75%), and villous adenoma (83.33%), in addition to that no nuclear or cytoplasmic reaction were showed in any case of control group.

In view of the many cellular modulatory properties of this virus, our data justify further studies to establish whether HCMV interfere with the pathogenesis of colorectal adenocarcinoma.

Key words: Thi-Qar, Human Cytomegalovirus, colorectal adenocarcinoma, Immunohistochemistry (IHC)

ملخص البحث

يعتبر سرطان القولون والمستقيم ثالث السرطانات شيوعا عند الرجال والنساء وهو الثاني من السرطانات المسببة للوفاة.حديثا اكتشف إن بروتينات الفيروس المضخم للخلية البشرية (HCMV) موجودة بنسبة عالية في سرطانات القولون والمستقيم البشرية.. هذا الاكتشاف رفع الفرصة للفيروس HCMV عند الإصابة الدائمة في إحداث المسالك السرطانية التي تحصل في سرطان القولون والمستقيم. إن الدراسة الحالية بحثت في إمكانية هذا الفيروس التدخل في حدوث وتطور سرطان القولون والمستقيم بواسطة الكشف عن بروتين (Pp65) للفيروس المضخم للخلية البشرية HCMV في الخلايا الظهارية لسرطان القولون والمستقيم باستخدام تقنية التصبيغ الكيميائي النسيجي المناعي (IHC) والنسيج الطبيعي الذي اخذ من الأنسجة المحيطة بالسرطان الخبيث كمجموعة سيطرة.في هذه الدراسة)لذلك أخذنا عينات على شكل قطع برافين لسرطان القولون والمستقيم والورم ألغدي الزغابي ٥٥ عينة صنفت إلى ثلاثة مجاميع :الورم الخبيث ،الورم ألغدي الزغابي ،ومجموعة السيطرة. كل

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للكشف عن بروتين الفيروس IHCالمجاميع خضعت لتقنية التصبيغ الكيميائي النسيجي المناعي Pp65 . باستخدام أجسام مضادة أحادية النسل لبروتين HCMVالمضخم للخلية البشرية ، لوحظ وجود تفاعل ايجابي بنوعية السايتوبلازمي و النووي لبروتينات الفيروس في IHCنتائج ، (83.33)الخلايا الظهارية لسرطان القولون والمستقيم(٧٥,٧٥%) ومجموعة الورم ألغدي الزغابي ولم تلاحظ هذه التفاعلات في مجموعة السيطرة.بملاحظة بعض الخصائص التغيّرية الخلوية لهذا الفيروس، فإن نتائجنا تفتح المجال لدراسات أوسع من اجل إثبات تدخل الفيروس المضخم للخلية في نشوء وتطور سرطان القولون والمستقيم.HCMVالبشرية

Introduction

The dietary, chemical and genetic factors have been studied most in relation to large bowel adenocarcinoma.

Colorectal carcinoma is the third most common cancer and the second most frequent cause of cancer death in many industrialized countries (1).

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 (2). HCMV may play the role of a non obligate either direct or indirect co-factor for tumorigenesis, e.g. by blocking apoptosis, which may be an essential requirement for tumor progression .Due to the stimulation of growth factors, HCMV may modulate the malignant potential for tumor cells. It has become apparent that HCMV manipulation of the host cell cycle as well as the immune response promotes the replication and propagation of the virus.

The ability of HCMV to modulate component of the host immune system and the response to infection most likely contributes to the pathology association with this virus (3). Recently, it was hypothesized that HCMV might be associated with colorectal carcinoma progression; numerous studies had linked HCMV infection with colorectal carcinoma.

Materials and Methods

Patients and Controls

This study was conducted from April to December 2005; paraffin-embedded blocks were collected from pathological archive of a private Lab., Kadhimya Hospital, Yarmok Hospital and GIT centre in Baghdad.

The study include 55 cases were screened for the presence of HCMV proteins using IHC, 33 cases from group A (adenocar-cinoma), 12 cases from group B (villous adenoma) and 10 cases from group C (control group) were screened for HCMV early protein (pp65).

Materials and methods

The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary antibody will form a complex with the peroxidase–conjugated streptavidin, and by adding the substrate, which contains 3, 3'–diamino-benzidine (DAB) in a chromogen solution, a brown colored precipitate will form at the antigen site.

Dakocytomation LSAB2 system-Horse Radish Peroxidase (HRP) code Ko673 immunohistochemistery detection kit

Mouse monoclonal antibody (anti-pp65) as an early gene of the cytomegalovirus (Biogenex, USA)

DAKO anti-CMV that contain two mouse monoclonal antibodies, DDG9 and CCH2, recognizing CMV immediate early gene and early gene products (pp76, pp43, respectively) (Dakocytomation, Denmark).

De-ionized distilled water.

Distilled water

Phosphate -buffered saline tablets (PBS tabs) (Flow Laboratories, U.K.).

Absolute ethanol (Fluka), 95%, 85% and 75%

Xylene (Analar, England)

Aquous mounting media (Chemicon, AM 100)

Antigen retrieval solution (DAKO, Denmark)

Positively charge microscope slides (Fischer scientific, USA)

Stirrer

Piston-operated pipette and tips (50µl, 10µl, 100ul. and1000 µl)

Graduated cylinders

Glass staining jars

Slide holder (10-slide each).

Hot air oven (Gallant Kamp Oven BS, England)

Timer with alarm

Tissue paper (Belgium, Hungary and Italy)

Pap pen (Dakoccytomation, Denmark A/S)

Incubator (Memmert, Germany)

Sterile gloves

Cotton swap

Cover slips (Marienfeld, Germany)

Humidity chamber

Microtome (Microm international, Germany)

Water bath (Memmert, Germany)

Mayers heamatoxylin (Biocare medical, Concord)

Immunohistochemistry procedure:

The procedure applied according to (4; 5; 6).

Deparaffinizationthe tissue sections: paraffin-embedded sections were placed inside hot air oven at 70°C, 20 minutes, then immediately dipping in xylene and ethanol containing jars as the following:

***xylene for 5 minutes**

***absolute ethanol for 5 minutes**

95% ethanol for 5 minutes

85% ethanol for 5 minutes

75% ethanol for 5 minutes

Later all slides were washed in distilled water for 5 minutes, then drying and blotting gently.

Preparing retrieval solution 10 x by using cobllin jars then put these jars in water bath at 90°C for 20 minutes, left cobllin jars to cool for 20minutes.

The tissue sections were surrounded with a circle drawn with a pap pen and allowed to dry for 2 minutes at room temperature. The hydrophobic barrier made by the pen retains aqueous solutions within a defined area, eliminating the use of excess reagents.

Wash the slides with PBS.

Drying and blotting, then put all slides in humidity chamber.

Aqueous 3% H2O2 (2 – 3 drops) was applied onto the tissue to cover the whole specimen to block endogenous peroxidase for 15 minutes (7).

Rinse the slides in PBS, then drying and blotting.

50 – 100 µl of diluted primary antibody was applied on the sections for about 30–60 minutes.

Apply1 – 2 drops of biotenylated link antibody (secondary antibody) to all sections for 10 – 15 minutes.

Rinse the slides in PBS, drying and blotting.

Apply 1 – 2 drops of streptavidin peroxidase (red) to cover the sections for 10–15 minutes.

Rinse the slides in PBS, drying and blotting.

Apply the prepared DAB-substrate chromogen solution by using provided transfer pipette; add enough drops to cover the sections for 10–15 minutes.

Rinse the slides in distilled water.

Dipping the slides in Mayer's heamatoxylin as a counter stain for 2 minutes

Rinse the slides in tap water.

Dipping the slides in the jars containing mixture of tap water (200 ml) and 2-3 drop of strong ammonia

Rinse the slides in tap water.

Dry the slides gently, then applied one drop of aqueous mounting media then put cover slip on the slides.

Leave the slides to dry, and then tested it.

Results

Clearly positive stains were appeared by IHC technique Group A (malignant group) 25 from the total number 33 (75.75%).Group B (benign group) 10 from total number 12 (83.33%), where as all cases involved with group C (control group) appeared negative. HCMV positive cases showed immunostaining of epithelial cells of colorectal adenocarcinoma and villous adenoma (Figure 1).

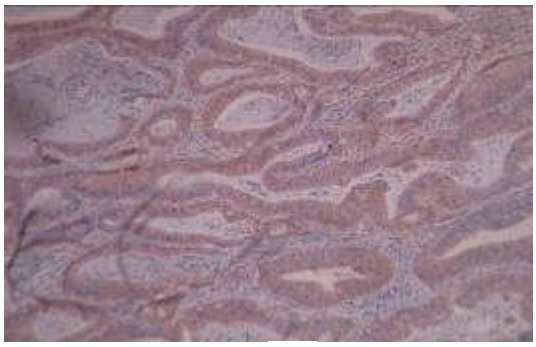
From the total of 45 cases (without the controls) of colorectal –tissue samples, there are 33 cases of colorectal adenocarcinoma (73.3%).

According to the grade of the cancer, these cases classified into 3 grades, GI, GII and GIII.

According to the stage of the cancer, the cases classified into 3 stages (according to the modified astler collar system that applied in the private lab.), B1, B2, and C1 (Table 1).

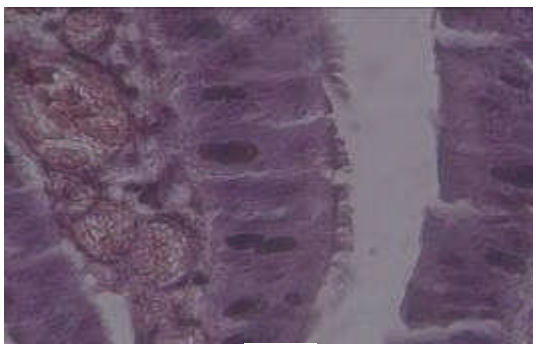
Figure 1) Immunohistochemistry staining of HCMV early protein (Pp65) (brown color) in:
A / colorectal adenocarcinoma, grade I, cytoplasmic reaction (x400)

B /colorectal adenocarcinoma, grade II, nuclear reaction (x800)
C and D /villous adenoma, cytoplasmic reaction (x400)

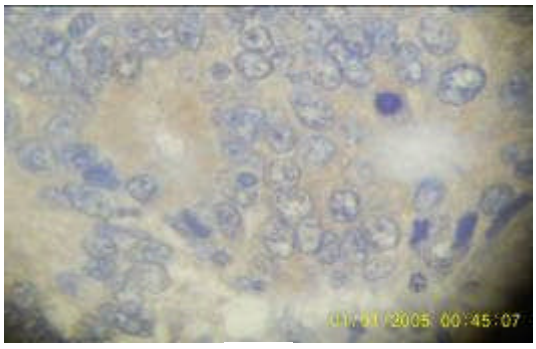


A

C



B



D

Discussion

Specific localization of HCMV proteins:

The current study demonstrates the presence of the HCMV proteins, 25 of 33 specimens (75.75%) in group A showed nuclear and cytoplasmic stained.

Also group B has nuclear and cytoplasmic positive stain in 10 of 12 specimens (83.33%).

Indeed, It was reported several years ago that HCMV infection results in the stimulation of cellular DNA synthesis (8), implying overriding of normal cell cycle control, this effect has been studied recently in greater detail in quiescent fibroblast cells that have been withdrawn reversibly from the cell cycle by serum deprivation or contact inhibition (9; 10). Also, HCMV infection has long been associated with the stimulation of host DNA, RNA, and protein metabolism in permissive as well as non permissive cells (11). Therefore ,the data presented here demonstrate an association of HCMV with colorectal tumorigenesis, some of HCMV proteins might have a mutagenic potential which might be expressed only transiently in host cells to induce mutations in cellular genes leading to oncogenic transformation (12), where as (13), found that the HCMV inhibits the growth of human foreskin fibroblast cells by 12 hours after infection, they demonstrated that HCMV does not arrest cell cycle progression at a single point but at least two blockage occur, one of which in G1 phase of the cell cycle. Various studies in vitro have demonstrated that the gene products of HCMV are capable to modulating the cell cycle progression and apoptosis by a number of important host genes (14; 15; 16; 17). Furthermore, HCMV infection stimulates cellular DNA synthesis and causes chromosomal damage, early after infection, HCMV induced elevated levels of cyclin E, cyclin E-associated kinase activity and two tumor suppressor proteins p53 and RB, the steady-state concentration of RB continued to rise throughout the infection with most of the protein remaining in the highly phosphory-lated form, at early times, HCMV infection also induced cyclin B accumulation, which was associated with a significant increase in mitosis –promoting factor activity as the infection processes (18). The phospho-protein pp65, also termed (pp UL83) (19), lower matrix protein (20), gp64 (21), p66 (22), pk68(23), or ICP27 (24), of HCMV is abundantly synthesized during lytic infection in cultured fibroblasts (25), Several studies showed a link between the pp 65 and protein kinase activity that lead to increase the phosphorelation form of RB family, for example, William and colleagues used a monoclonal

antibodies directed against pp 65 then purified of this protein by electrophoresis confirmed that the kinase activity was associated with this protein(3), also kinase activity has been found associated with pp65 in several studies (26; 25). So that, we can conclude that the HCMV proteins are localized in the epithelial cells of colorectal adenocarcinoma and villous adenoma, the positivity of HCMV shows nuclear and/or cytoplasmic reaction according to the HCMV infection, so, the cases that revealed cytoplasmic and nuclear reaction because these cases were obtained in advanced stage of HCMV infection.

Table (1) Histopathological data according to the grade and stage of colorectal adenocarcinoma

Variables	Patients No.=33 (malignant group)		Age (years)	
Grade	33	73.3%	Mean range	
GI	1	3.03	40	40
GII	18	54.54	52	22-79
GIII	14	42.42	52	34-70
Stage				
B1	11	33.33	59	34-77
B2	17	51.51	57	26-75
C1	5	15.15	46	22-79

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