

Investigation of Human Cytomegalovirus pp43 & pp76 in colon adenocarcinoma Using Immunohistochemistry (IHC) technique.

-Atta Gitti Allawi / Consultant physician/ Dean of the college of Medicine.

-A.BassitA.Samad/Phd./Head of Microbiology Department .

- Ahmed M.A.Nazar /Msc.Virology

*College of Medicine /Wassit University,Iraq.

الخلاصة

بعض الدراسات الوبائية اقترحت احتمالية وجود علاقة بين الفيروس المضخم للخلية البشرية (HCMV) ومختلف السرطانات الخبيثة وخصوصا سرطان القولون.إن الدراسة الحالية بحثت في إمكانية تدخل هذا الفيروس في حدوث وتطور سرطان القولون بواسطة الكشف عن بروتينات الفيروس في الخلايا الظهارية لسرطان القولون باستخدام تقنية التصبيغ الكيميائي النسيجي المناعي (IHC) . لذلك أخذنا عينات على شكل قطع برفين لسرطان القولون والنسيج الطبيعي الذي اخذ من الأنسجة المحيطة بالسرطان الخبيث كمجموعة سيطرة . هذه العينات فحصت باستخدام (IHC) للكشف عن بروتينات الفيروس المضخم للخلية البشرية باستخدام نوعين من الأجسام المضادة وحيدة النسائل خليط من البروتين المبكر والبروتين المبكر الآتي (PP76,PP43) على التوالي . نتائج (IHC) ، لوحظ وجود تفاعل ايجابي بنوعية السايتوبلازمي و النووي لبروتينات الفيروس في الخلايا الظهارية لسرطان بنسبة (82.60 %) ولم تلاحظ هذه التفاعلات في مجموعة السيطرة . بملاحظة بعض الخصائص التغيرية الخلوية لهذا الفيروس ، فإن نتائجنا تفتح المجال لدراسات أوسع من اجل إثبات تدخل الفيروس المضخم للخلية البشرية (HCMV) في نشوء وتطور سرطان القولون.

Summary

Several studies have suggested a possible link between human cytomegalovirus infection and various malignancies particularly colon adenocarcinoma. The present study investigates whether HCMV participates in human colon tumorigenesis by the detection of HCMV proteins within epithelial cells of colorectal carcinoma using Immunohistochemistry (IHC) technique. formalin – fixed, paraffin – embedded specimens of adenocarcinoma, and normal tissues were obtained from the margins of the excision as a control, those specimens were tested by IHC to detect the presence of HCMV proteins using two types of mouse monoclonal antibodies as mixture of monoclonal antibodies to an early and immediate early proteins (pp43 & pp76, respectively). The results of IHC assay showed specific nuclear and cytoplasmic reaction of HCMV proteins within the epithelial cells of colon adenocarcinoma (82.60%), the study showed no nuclear or cytoplasmic reaction 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 colon adenocarcinoma.

Introduction

Most colon adenocarcinoma arise sporadically, about 5% are thought to be due to inherited predisposition syndromes. Despite present understanding of genetic alterations associated with progression of colon adenocarcinoma, results of epidemiological studies suggest that environmental factors and host immunological characteristics could contribute to initiation and progression of this cancer. Frequency of colon adenocarcinoma is ten times higher in developed countries than in some developing countries, and 3.6 times higher in immunosuppressed patients than in those who are not immunosuppressed (Parkin *et al*, 1993).

A 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 (Macswen & Crawford, 2003), human herpesvirus – 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). In immunocompromised patient with infection, focal colonic epithelial lesions can arise (Britt & Alford, 1996). Data suggest that gene products of HCMV promote mutagenesis, cell - cycle progression, angiogenesis, cell invasion and immune evasion (Cinatl *et al*, 1996; Doniger *et al*, 1999). Although there is no definitive evidence of the association of HCMV infection with human cancer, the oncogenic potential of HCMV has been well established by *in vitro* studies demonstrating the ability of UV-irradiated of infectious virus to transfer a variety of cells.

In human colonic adenocarcinoma cells, HCMV infection can only arise when these cells are in a specific state of differentiation, virus dose not spread from cell to cell, and productive infection is rare (Jarvis *et al*, 1999).

Material and Methods

The study included two groups :the first group included (23 cases) of colon adenocarcinoma, and the second group (8 cases) were the control group(margin of excision). All cases were screened for the presence of HCMV proteins using IHC 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' –diaminobenzidine (DAB) in a chromogen solution, a brown colored precipitate will form at the antigen site.

Materials & Equipments

- Dakocytomation LSAB2 system- Horse Radish Peroxidase (HRP) code Ko673 immunohistochemistry detection kit.
- DAKO anti-CMV that contain two mouse monoclonal antibodies ,DDG9& CCH2 ,recognizing CMV immediate early gene & 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 95%, 85% and 75%.
- Xylene.
- Aquous mounting media .
- Antigen retrieval solution .
- Positively charge microscope slides.
- Incubator.
- Humidity chamber.
- Microtome.
- Water bath.
- Mayers heamatoxylin (Biocare medical, Concord).

Methods

The following procedure was used according to the method of (Cobbs, *et al.* 2002)

- Paraffin-embedded sections were placed inside hot air oven at 70°C, 20 minutes, then immediately sub emerged in xylene and ethanol containing jars absolute,95% ,85%,75% ethanol.

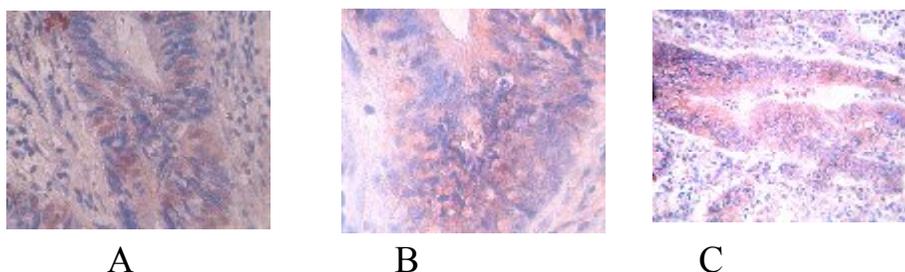
- Retrieval solution (10x) was prepared by using cobllin jars then put these jars in water bath at 90°C for 20 minutes, left cobllin jars to cool for 20minutes.
- The slides were washed with PBS, then drying and blotting.
- Aqueous 3% H₂O₂ (2 – 3 drops) was applied onto the tissue for 15 minutes.
- The slides were rinsed in PBS, then drying and blotting.
- 50 – 100 µl of diluted primary antibody was applied on the sections for about 30 – 60 minutes.
- One-two drops of biotenyated link antibody was added (secondary antibody) to all sections for 10 – 15 minutes.
- The slides were rinsed in PBS, drying and blotting.
- One- two drops of streptavidin peroxidase (red) were added to cover the sections for 10 – 15 minutes.
- The slides were rinsed in PBS, drying and blotting.
- The prepared DAB- substrate chromogen solution was used for 10 – 15 minutes.
- The slides in were rinsed distilled water.
- The slides were sub emerged in Mayer's heamatoxylin as a counter stain for 2 minutes.
- The slides were rinsed in tap water.
- The slides were sub emerged in the jars containing mixture of tap water (200 ml) and 2 -3 drop of strong ammonia.
- The slides were rinsed in tap water.
- The slides dried gently, then applied one drop of aqueous mounting media then put cover slip on the slides.
- The slides left to dry, and then tested it.

Results

Clearly positive stains were appeared by IHC technique, first group (malignant group) 19 from the total number 23(82.60%), where as all cases involved with the second group (control group) appeared negative. HCMV positive cases showed immunostaining of epithelial cells of colon adenocarcinoma (Figure 1).According to the grade of the cancer, these cases classified into 3 grades, GI, GII & 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, & C1 (Table 1).

Table1:Histopathological data according to the grade and stage of colon adenocarcinoma.

Variables	Patients No.=23(malignant group)		Age (years)	
Grade	23	74.19 %	Mean range	
GI	1	4.34	40	40
GII	15	65.21	52	22-79
GIII	7	30.43	52	34-70
Stage				
B1	5	21.73	59	34-77
B2	7	30.43	57	26-75
C1	11	47.82	46	22-79



(Figure 1):Immunohistochemistry staining of HCMV mixture of early and immediate early proteins (brown color) in:
A-colon adenocarcinoma, grade I, cytoplasmic and nuclear reaction (x400).
B-colon adenocarcinoma, grade II, cytoplasmic and nuclear reaction (x400).
C-colon adenocarcinoma, grade III, cytoplasmic and nuclear reaction (x400).

Discussion

The detection of HCMV DNA and antigen 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).However ,it is unlikely that HCMV directly causes cancer since the low incidence observed for each of the cancers linked to the virus which don't reflect the ubiquitous nature of HCMV within the population .It has been suggested that HCMV may act as a co etiologic agent in the development of tumors through a hit and run mechanism ,HCMV promote cellular transformation by causing genetic

instability or by preventing cells from undergoing apoptosis (Shen *et al*,1997;Lukac&Alwine,1999).

Our study would explain the presence of HCMV proteins within the colon adenocarcinoma, 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. Recently ,significant amount of HCMV proteins have been detected in prostate carcinoma lesions (Samanta *et al.*, 2003) ,and also found to be localized specifically in neoplastic cells in human colorectal polyps and adenocarcinoma (Cobbs, 2002), Olarank and colleagues, examined a large number of colon hyperplastic polyps, adenoma and adenocarcinoma of the colon for the presence of HCMV proteins by IHC assay using mixture of two mouse monoclonal antibodies,DDG9 & CCH2 ,pp76 ,pp43 ,respectively (Akintola – Ogunremi *et al* ,2005). The same antibodies from Dakocytomation were used to detect the expression of the HCMV proteins in benign and malignant lymphoproliferative diseases (Luppi *et al* ,1998).

The oncogenic potential of HCMV is well established in vitro ,HCMV inhibits apoptosis (Cinatl *et al.*, 1998), induces malignant transformation ,and dysregulates key cellular pathways involved in mutagenesis , angiogenesis (Cinatl *et al.*,1999) , and evasion of immune recognition (Beck *et al.*, 2003).

However, although several studies indicate that HCMV could facilitate tumor progression, there is still no definitive evidence of a causal link between HCMV infection and human cancer, one reason for this lack of evidence may be due to the limited availability of adequate cell culture models, HCMV – specific components are not detectable after long- term subcultures of tumor tissues (Rosenthal & Choudhury, 1993). Our study investigates the possible association between HCMV and at least colon adenocarcinoma , but there is a special attention devoted to investigate the interaction between the HCMV and cell cycle regulatory proteins such as cyclin ,E2F p53 and PRB related proteins ,which may disrupt cell cycle regulation and lead to abnormal cell proliferation ,the hallmarks of HCMV infection are the massive induction and dysregulation of tightly regulated transcription factors and the activation of proteins associated with proliferation ,this virus –mediated cellular events are crucial in initiating the viral pathogenesis associated with a wide range of clinical manifestations (Huang & Kowalik, 1993).

According to these results we can conclude HCMV proteins are localized in the epithelial cells of colon adenocarcinoma. Although there is no strong evidence indicating that the HCMV involved in the

colon tumorigenesis but the detection of HCMV proteins within the colon cancer reflects cell permissiveness to the viral replication.

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