Original Article

Detection of Human Cytomegalovirus in Colorectal Adenocarcinoma by In Situ Hybridization Technique

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Summary:

Background: Human Cytomegalovirus (HCMV) infects a wide range of human cells, including colonic epithelial cells that give rise to adenomas and adenocarcinomas. Persistent productive infection of tumor cells is essential for oncomodulation by HCMV.This study aimed to detect HCMV matrix protein using in situ hybridization technique (ISH) in colorectal adenocarcinoma compared to normal colon tissues, and to the presence of cytomegalovirus inclusion bodies in infected colorectal carcinomas.

Patients and methods: Twenty six of colorectal adenocarcinomas were obtained in paraffin-blocks compared to 10 normal colon specimens which were age and sex matched as control group. Detection of HCMV was obtained by in situ hybridization technique.

Results: The biotinylated probe specific for DNA encoded HCMV-matrix protein showed hybridization with nucleic acid in 20 cases out of (26) of colorectal adenocarcinomas representing (76.9%) compared to normal colon tissue which revealed no hybridization signals. Moderate to high scoring signals were detected in moderate to poorly differentiated groups. Inclusion bodies were detected in 11 (42.3%) cases with positive hybridization signals.

Conclusion: HCMV may play a role in the colorectal adenocarcinoma pathogenesis. In situ hybridization test are considered the most sensitive and specific tools for detection of HCMV DNA in tissues. Epidemiological, histopathological identification of cytomegalic inclusion bodies and molecular studies are necessary to confirm the association of HCMV and colorectal tumorogenesis in Iraqi population.

Keywords: HCMV, colorectal adenocarcinoma, ISH, inclusion bodies.

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Introduction:

Human Cytomegalovirus is ubiquitous herpesvirus that leads to lifelong persistent infection. The frequency of infection ranges from 50% to 90% in general adult population, and varies with socio-economic level and to some extent, geographic location (1). CMV is common causative of gastrointestinal tract diseases in immunodeficient patients leading to high morbidity and mortality, focal colonic epithelial lesions can arise (2).Numerous reports on the detection of viral DNA, mRNA and/or antigens in tumor tissues, as well as seroepidemiological evidence, have implicated HCMV in etiology of several malignancies, including colon carcinoma, cervical carcinoma, malignant glioma, prostate adenocarcinoma, and pediatric malignancies such as Wilm's tumor and neuroblastoma (3, 4, and 5). Data suggest that gene products of HCMV can promote
Mutagenesis, cell-cycle progression, angiogenesis, cell invasion, and immune evasion (6, 7). However, the relation of CMV infection with colorectal cancer is still unclear and divergent. Detection of infectious agent in human cancer might have important implication in cancer treatment and prevention (3).

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Patients and methods:

Patients: This study was designed as retrospective research. A formalin fixed paraffin embedded tissue from 26 surgical biopsy specimens of colorectal adenocarcinoma and 10 normal colons were collected from record of pathological archives of Teaching Laboratories of Medical City Hospital during a period of October 2007 – March 2008. The diagnosis of these tissue blocks were based on records. A pathologist reexamined all cases to confirm diagnosis following trimming process of these tissue blocks. No one of all patients was immunocompromized.

The age of these patients ranged from 37-70 years with (10 cases were males and 16 cases were females), compared to 10 normal colon specimens which were age and sex matched with study group as control group. Methods:Detection of HCMV by ISH-kit (Maxim Biotech USA) was performed on 4 µm paraffin embedded tissue section using a biotinylated long DNA probe encoding for CMV matrix protein (Maxim biotech USA cat No. 1H 60043). All the method was conducted according to manufacturing company leaflet (DNA probe hybridization/ detection system in situ kit 2003).Positive control reactions are performed by replacing the probe with biotinylated house keeping gene probe. Negative control was obtained by omitting the probe from hybridization

buffer. Color development can be monitored by viewing the slides under the microscope. A blue colored precipitate will form at the site of the probe in positive cells.

Statistical Analysis: Statistical significance was assessed using the chi-square test, Fisher's exact test. Correlation is considered significant when $p<0.05$. Results were expressed as percentage, range and mean ± S.D.

Results:

Table (1) shows the selected age, sex of colorectal adenocarcinoma patients compared to normal colon as control group included in ISH experiment using biotinylated probe for DNA encoding CMV matrix protein .The results were interpreted as directed by manufacturing company

Positive hybridization signals were detected in form of blue granules located in positive cells (Fig.1A, B). Twenty cases of colorectal adenocarcinomas revealed positive hybridization signals representing (76.9%) which is statistically significant (P=0.0002) (Table 2A). These include 3 (11.5%) of well differentiated, 11 (42.3%) of moderate, and 6 (23.1%) of poorly differentiated, table (2B).

None of normal healthy control group revealed hybridization signal (fig.1C). Scoring of positive signals was put according to number of granules per high power field and as follows:

Score 1 (low): with 1-3 granules/10HPFs

Score 2 (moderate): with 4-6 granules/10HPFs

Score 3 (high): with more than 6 granules/10HPFs

(Table-1) Distribution of patients with colorectal adenocarcinomas and the healthy control according to the age and sex

Study Groups		Colorectal adenocarcinoma	Healthy control
No.		26	10
Age/years	range	37-70	40-65
	mean	55.27	54.4
	SD	±9.88	±8.80
Sex	male	10	5
	female	16	5

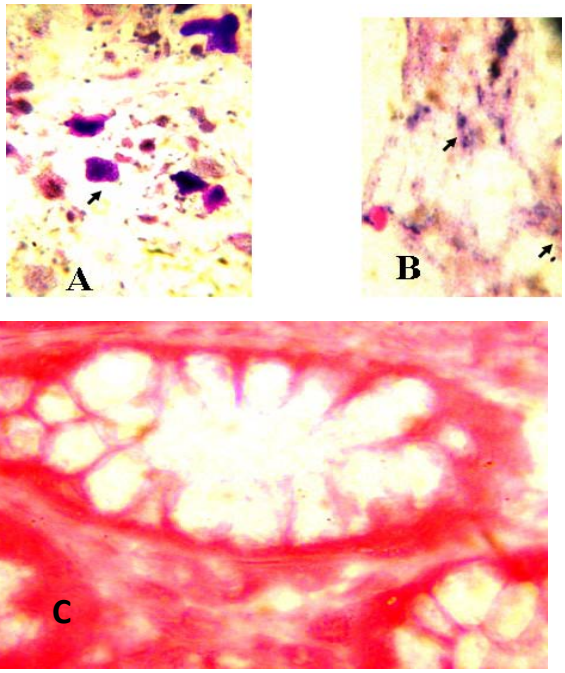

Figure (1). ISH for HCMV DNA encoding Matrix protein using biotinylated DNA probe. Colorectal adenocarcinoma show positive hybridization signals stained by *BCIP/ NBT and counterstained by Eosin (A, B) .Normal colon tissue show no hybridization signals (C). (X1000)

* 5-Brom-4 Chloro-3 Indolyl Phosphate/ Nitro Blue Tetrazolium

(Table-2A) ISH results among study groups

Study groups		ISH results		Total
		Positive	Negative	
Colorectal adenocarcinoma	N	20	6	26
	%	76.9	23.1	100
Normal colon	N	0	10	10
	%|	0	100	100
Total	N	20	16	36
	%	55.6	44.4	100

P=0.0002

(Table 2B) Distribution of Colorectal adenocarcinoma by degree of differentiation & ISH results.

Degrees of differentiation of colorectal adenocarcinoma		ISH results		Total
		Positive	Negative	
Well	N%	3 (11.5)	4 (15.4)	7 (26.9)
Moderate	N%	11 (42.3)	2 (7.7)	13 (50)
Poorly	N%	6 (23.1)	0 (0)	6 (23.1)
Total N (%)		20 (76.9)	6 (23.1)	26 (100)

(Table-3) Scoring of Hybridization with positive ISH results among colorectal adenocarcinomas

ISH Score	Degree of differentiation			Total
	Well	Moderate	poorly	
Low 1-3	1	0	0	1
Moderate 4-6	2	5	0	7
High >6	0	6	6	12
Total	3	11	6	20

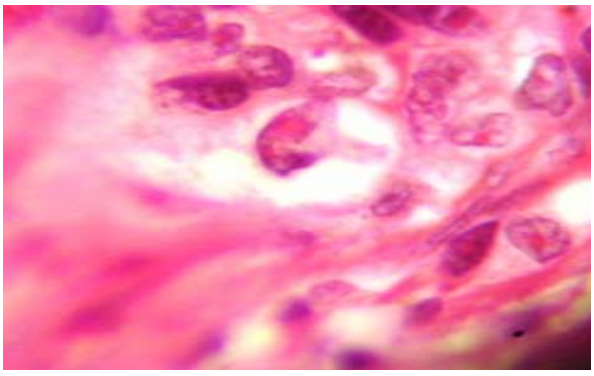

Figure (2). Presence of eosinophilic inclusion bodies (H and E) (X1000)

(Table-4)Detection of HCMV nucleic acid in relation to the presence of the intracellular inclusion bodies

Inclusion bodie	ISH-Results		Total No.
	Positive No. (%)	Negative No. (%)	
Present	11(42.3)	0	11(42.3)
Absent	9(34.6)	6(23.1)	15(57.7)
Total No.	20(76.9)	6(23.1)	26(100)

P =0.02

Table (3) revealed moderate positive ISH scoring in (5) moderate differentiated cases and high score were detected in (6) moderate differentiated and also in (6) poorly differentiated colorectal adenocarcinomas. Low score was detected in only one case of well differentiated group. There was insignificant correlation between scoring signal and degree of differentiation.

Histological re-evaluation of different H & E stained section included in this study.

Inclusion bodies were in form of single large red intranuclear or intracytoplasmic one (fig 2). All cases with positive inclusion bodies 11(42.3%) showed positive hybridization signals. These results were statistically significant (P =0.021) as shown in table 4.

Discussion:

Colorectal carcinoma is the third most common cancer, and is the second most frequent cause of death in many industrialized countries (8). Most colorectal cancer is sporadic, and only about 5 % are thought to have genetic basis. Results of epidemiological studies suggest that environmental factors and host immunological characteristics could contribute to initiation and progression of the cancer [8, 3]. The possible association of CMV with human colorectal adenocarcinomas was reported first in 1978 by Huang and Roche (9), who detected CMV DNA in 4 of 7 colonic adenocarcinomas by membrane complementary RNA-DNA hybridization. It is interesting that CMV DNA also was detected in 1 of 2 cases of familial adenocarcinomas polyposis but not in normal colonic tissues from the same patients or control cases of Crohn's disease. However several subsequent studies performed on small samples using various techniques have shown inconsistent results (10, 11). The data presented herein demonstrate that

HCMV DNA of late gene that encodes matrix protein product can be found and localized in epithelial cells of human colorectal adenocarcinomas, representing 76.9% using ISH technique. Matrix protein is the most abundant structural protein involved in the synthesis of virion (1). Seroepidemiological study among healthy population demonstrate that CMV is endemic in Iraq and that seroprevalence of CMV is high (95.4%) in different age groups (12). Intense blue granules (ISH-score) increased proportionally with the degree of differentiation (moderate-poor). This may indicated that long term persistent HCMV infection and expression in dysplastic colonic epithelial cells could be important in promotion of oncogenic events directly implicated in malignant progression (3). The present data support the earlier observation and also comparable with the study of Harkins *et al* in 2002 (3) who detect CMV protein and nucleic acid of CMV in 82% of polyps cells and 92% of adenocarcinomas but non in adjacent normal tissue using immunohistochemistry analysis with monoclonal antibodies for HCMV immediate early gene 1E1-72, HCMV pp65 delayed early tegument protein, in situ hybridization and PCR with DNA sequencing. In contrast our data disagree with other study which showed no direct immunohistochemical and molecular evidence of CMV with human colorectal tumorogenesis compared to control (11, 13), and with other study which suggest that the frequency of CMV infection in ulcerative colitis was significantly higher than that observed for colorectal cancer (2). These results, however, do not exclude the possibility of a "hit-and-run" hypothesis that claims a virus can mediate cellular transformation through an initial "hit" while maintenance of transformed state is compatible with loss "run" of viral molecules (13, 14). Recently, the term oncomodulation has been proposed to express the ability of HCMV to modify tumor cell biology, a phenomenon that is independent from transformation. This may occur through the production of viral proteins that effect cellular differentiation, gene expression, DNA replication and cell cycle progression (15). The virus gene products immediate early protein 1E-72 and 1E2-86 can dysregulate cell cycle check points by interaction with TP53 and retinoblastoma tumor suppressor proteins and downstream pathway, also these proteins can block apoptosis mediated by tumor necrosis factor α (3, 16). In addition the levels of proto-oncogen c-myc, c-fos and c-jun are also rapidly upregulated following infection of cells by HCMV [(17)]. The HCMV infection has also been implied to increase tumor-invasiveness through increased migration of infected tumor cells (15). The virus leads to immunosuppression that may debilitate the host immune response against growing tumor. Furthermore, the HCMV protein UL16 may confer resistance against cells lysis mediated by NK cells and T cells. Therefore, HCMV infection of tumor cells may prevent the desired effects of chemotherapy and immunotherapy and should therefore be considered in the treatment of these patients (7). In infected intraphase like cells the immediate early 1E1/ 1E2 antigen are confined to nuclei bounded by an intact nuclear membrane, likely as the ultimate site of virion tegumentation and envelopment (18). Moreover, this structure is visible as cytoplasmic inclusion and functioning as a gathering site for cellular organelles, dense bodies, virions, and other virus-like particles at late times post infection (19, 20). In present study intracytoplasmic and intranuclear inclusion bodies were observed in more than half of cases of colorectal adenocarcinoma with different degree of differentiation which is comparable to other study in Iraq which detected HCMV in different types of malignant gliomas using in situ hybridization test as well observation of inclusion bodies in more than half of cases (21). As predicted from previous studies in that CMV infection was associated with the initiation of mitotic event in the host cells and with generation of abnormalities in the process of centrosomes duplication, spindle assembly and chromosome condensation (22). Accordingly, the use of conventional H & E stain may give a clue to HCMV infection, but it can not exclude the possible role of other viruses. However confirmation of infection requires the use of specific ISH or immunohistochemistry technique. In this study it was found that all cases with inclusion bodies were positive for HCMV by ISH. A further research conduct a large cohort study recruiting a large number of colorectal cases using DNA sequencing and detection of viral proteins may leads to better understanding of the biology of this malignancy.

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