Detection of Cytomegalovirus DNA in Colorectal Tissue from Swedish and Vietnamese Patients with Colorectal Cancer

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Abstract. *Background: Human cytomegalovirus (HCMV) has been implicated as a factor, which might be associated with colorectal cancer (CRC) progression. Data from studies with HCMV-infected tumour cell lines have highlighted an oncomodulatory potential of HCMV. In the present study, we aimed to evaluate the prevalence of HCMV DNA in CRC tissue compared to matched normal tissue, and its association with clinical factors. Patients and Methods: We used quantitative real-time polymerase chain reaction assay to detect HCMV DNA in 202 cancerous and paired normal tissue from Swedish (n=119) and Vietnamese (n=83) CRC patients. Results: Overall, the HCMV DNA rate was significantly higher in cancerous in relation to paired normal tissue. Furthermore, a significantly higher frequency (39.8%) of HCMV DNA was observed in cancer tissues from the Vietnamese patients compared to the Swedish patients (15.1%). The prevalence of HCMV DNA in CRC tissue of 50% of those with disseminated disease tended to be higher compared to those with localized disease, with a prevalence of 33.3% in Vietnamese patients. Conclusion: Our observations indicate that the prevalence of HCMV DNA differs significantly between cancer and matched normal tissues. Thus, these data support a possible role of CMV in CRC. Moreover, we noted differences between Swedish and Vietnamese patients, indicating a role of ethnicity.*

Human cytomegalovirus (HCMV) is a member of the herpesvirus family and establishes a life-long infection. HCMV manifests itself in its host depending on the immunological status and underlying condition. In a healthy population, a primary HCMV infection is often asymptomatic but may cause morbidity and mortality in immunocomprised patients, including individuals with AIDS, and organ transplant recipients (1). The frequency of infection ranges from 50% to 100% among different populations and varies according to geographic location and socioeconomic level (2-5). More than 70% of Swedish blood donors have been shown to be HCMV-seropositive and the prevalence increases with age (6, 7). The HCMV seroprevalence tends to be highest in Africa and Asia and lowest in Western Europe and the United States (2-5).

Data from studies with HCMV-infected tumour cell lines have highlighted an oncomodulatory potential of HCMV. This role of HCMV was defined as the ability of the virus to catalyze the oncogenic process associated with signal pathways and transcription factors (8, 9). There are no data supporting the clinical relevance of HCMV-induced oncomodulation in colorectal cancer (CRC). However, the presence of HCMV DNA and HCMV protein in CRC tissue has led to the assumption that an association exists between HCMV infection and CRC progression. Some studies have shown a possible association, while other studies have not been able to confirm any association (10-14). Interestingly, HCMV can interfere with cell control mechanisms through different pathways, including activation of oncogenes such as v-myc avian myelocytomatosis viral oncogene homolog (*MYC*), v-junsarcoma virus 17 oncogene homolog (*JUN*), murine osteosarcoma viral oncogene homolog (*FOS*) and *P53* (8, 9, 14). These pathways are highly associated with the development of CRC (15-18). Moreover, CRC-related events such as angiogenesis and apoptosis have been shown to be affected by HCMV infection, which leads to increased angiogenesis and inhibition of apoptosis (9, 19, 20). Recently it was shown that polymorphisms of cytokine genes affect reactivation of HCMV in patients with cancer (21). These findings may further support a link between HCMV and CRC as inflammation affects carcinogenesis.

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Key Words: Colorectal cancer, cytomegalovirus, viral DNA, Vietnamese, Swedish.

The present study was conducted to assess the rate of HCMV DNA in cancerous tissue compared to paired normal tissue and to evaluate its association with clinical factors. Moreover, a possible link to ethnicity was evaluated by comparing results in Swedish and Vietnamese patients with CRC.

Patients and Methods

Patients and tissue sampling. This study comprised of consecutive patients with CRC from southeastern Sweden and from northern Vietnam. Tissue and blood samples were collected when the patients underwent surgical resections for primary colorectal adenocarcinoma at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden and the National Cancer Hospital, Tamhiep, Hanoi, Vietnam. Clinicopathological characteristics of the patients were received from surgical and pathological records. Tumour tissue and adjacent normal tissue (about 5 cm from the tumour) from each patient were excised and immediately freshly frozen at –80˚C until analysis.

The Swedish patients (n=119) comprised of 68 males and 51 females, with a mean age of 69 (range=29-90) years. The tumours were located in the colon in 64 cases and the rectum in 55, and were classified according to the American Joint Committee on Cancer (AJCC) classification system (22) : Stage I in 21, II in 38, III in 41 and IV in 19. The Vietnamese patients (n=83) comprised 43 males and 40 females, with a mean age of 57 (range=26-95) years and tumours were classified as stage I in 25, II in 26, III in 29 and IV in 3. The tumours were located in the colon in 40 cases and the rectum in 43.

The investigation was approved by the Ethics Committee at the Faculty of Health Sciences Linköping, Sweden (Dnr. 98113).

Plasma samples and assay of HCMV serostatus. Blood samples from 116 and 36 of the Swedish and Vietnamese patients, respectively, were available for serological analysis. HCMV IgG antibodies were tested by enzyme-linked immunosorbent assay, Architect Anti-CMV-IgG (Abbott Laboratories, North Chicago, IL, USA). Antibody levels over 6 arbitrary units per ml (AU/ml) were considered positive.

Quantitative real-time polymerase chain reaction (PCR) assay. DNA was isolated from all tissues using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Purified DNA was analysed using the *artus* CMV TM PCR kit (Qiagen) for the specific amplification of a 105 bp region of the HCMV genome according to the manufacturer's instructions. The amplification was set up in a total volume of 50 μl containing 20 μl of extracted DNA and >5 copies were considered positive. The levels of HCMV DNA from the tumour and paired normal tissue were expressed as copies per microgram of total DNA (copies/μg).

Statistical analysis. Differences in the rates of HCMV DNA in samples were analyzed using the Chi-square test. Differences in serological and HCMV DNA data were determined by the Mann-Whitney *U*-test. All calculations were performed on a PC using SPSS for Windows computer package (Rel. 14.0; SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered significant.

Results

HCMV antibody data. In a total of 116 Swedish patients with CRC, 70.7% (82/116) had HCMV IgG antibodies in their plasma. In 46 patients, we found a range of 8-244 AU/ml and in 36 patients the level was >250 AU/ml. All tested Vietnamese patients (n=36) were seropositive, with a range of 19-244 AU/ml in 19 patients and >250 AU/ml in 17 patients. Overall, the Vietnamese patients had a significantly ($p<0.001$) higher proportion of seropositivity. No association between level of HCMV antibody and clinicopathological parameters of CRC was found.

HCMV DNA data. All Swedish patients with detectable HCMV DNA in colorectal tissue were seropositive for HCMV, and HCMV DNA in cancer tissues was detected in 18 out of 119 (15.1%) patients and in two matched normal tissues. In the Swedish seropositive group, the HCMV DNA-positive rate was 21.9% (18/82). A significantly ($p<0.001$) higher rate, 39.8% (33/83), of HCMV DNA-positive cases was recorded in cancerous tissues from the Vietnamese patients compared to Swedish patients (15.1%). Similarly, the rate of positivity for HCMV DNA was significantly ($p<0.01$) higher in normal tissue in Vietnamese (n=10) compared to the Swedish cases (n=2). Referring to the Swedish seropositive group with an HCMV DNA rate of 21.9% (18/82) in cancerous tissue, we also found that this differs significantly ($p=0.013$) from that of the Vietnamese patients, assuming a seroprevalence of 100% in these patients.

In the assessment of the HCMV DNA level, no significant difference was found in cancer tissues in Swedish patients (median=27, range=3-1833 copies/μg DNA) in comparison with that in Vietnamese patients (median=20, range=2-3044 copies/μg DNA).

To assess the HCMV DNA results according to the clinicopathological characteristics, all patients were divided into subgroups according to gender, tumour site and stage. With regard to disease stage, the patients were divided into two subgroups of stage I+II (localized disease) and stage III+IV (disseminated disease). The prevalence of HCMV DNA in CRC tissue of 50% (16/32) in those with disseminated disease tended to be higher ($p=0.13$) compared to those with localized disease, with a prevalence of 33.3% (17/51) in Vietnamese patients (Table I).

No association between the level of HCMV DNA and clinicopathological parameters of CRC patients was found. Furthermore, no significant differences within each ethnic group were noted regarding clinical factors.

Discussion

Current knowledge regarding the role of HCMV as a prognostic marker in CRC is controversial. It has been speculated that HCMV contributes to oncogenesis by a 'hit and run' mechanism but there is no conclusive evidence that the virus has an oncogenic role because normal cells do not seem to be transformed after infection (8, 9). Some studies including

Table I. *Rate (%) of HCMV DNA detection in tissue from colorectal carcinoma according to patient characteristics in Swedish and Vietnamese patients.*

	Variable Swedish patients (n=119)	Vietnamese patients (n=83)
Gender		
Male	16.1 (11/68)	46.5 (20/43)
Female	13.7 (7/51)	32.5 (13/40)
Tumour site		
Colon	10.9 (7/64)	35.0 (14/40)
Rectum	20.0 (11/55)	44.2 (19/43)
Stage		
I+II	13.6 (8/59)	33.3 (17/51)*
III+IV	16.7 (10/60)	50.0 (16/32)*

No significant difference in any of the categories. *The prevalence tended to be higher (p=0.13) in disseminated (stage III+IV) compared with localized (stage I+II) disease for Vietnamese patients.

analyses of HCMV DNA or proteins have shown a possible association with CRC, while other studies have not been able to confirm any association (10-14). In this study, we analyzed HCMV DNA in a considerable number of lysates prepared from cancer and matched normal tissue in Swedish and Vietnamese patients with CRC. We found in both Swedish and Vietnamese patients that the rate of HCMV DNA was significantly higher in cancerous in relation to paired normal tissue. Moreover, in cancerous and matched normal tissue from Vietnamese compared to Swedish patients revealed them to have a significantly higher proportion of HCMV DNA. Recently, comparable rates of HCMV DNA were found in Asian patients with CRC (23). It was notable that the prevalence of HCMV DNA in cancerous tissues tended to be higher in those with disseminated disease compared to those with localized disease within the Vietnamese patients. Our results reflect a clear accumulation of HCMV DNA in cancer tissue compared to normal tissue, and also a difference that may be linked to ethnicity.

The location of viral latency is not fully-characterized but mononuclear cells have been implicated as one of the most likely cell types harboring HCMV genomes (1, 14, 19). The underlying mechanism that controls the influx of HCMV into cancerous tissue is not yet understood. A restrained or an altered immunological response could contribute to the local accumulation of HCMV. Cytokines play a multifaceted role in local immunoregulation, which impacts on carcinogenesis (24-27). The higher rate of HCMV DNA in cancerous tissue compared to normal tissue could be due to the influx of leukocytes such as monocytes/macrophages harboring latent HCMV DNA. These cells migrate under the influence of chemotactic cytokines (chemokines) to the tumour and might thus locally increase the presence of HCMV DNA.

Recently, it has been reported that polymorphism of cytokine genes affect reactivation of HCMV in patients with cancer (21). One genotype of interleukin-2 (IL-2) was shown to have a protective effect against HCMV reactivation, whereas IL-4 and transforming growth factor beta-1 (TGFβ1) genotypes increased the risk of HCMV reactivation. Referring to these findings, the genetic background of the host could affect the local tissue environment and thereby influence HCMV reactivation.

The rate of HCMV DNA in the normal tissue of Vietnamese patients was significantly higher than that of Swedish patients. One can speculate as to whether patients with HCMV DNA in normal tissue have different disease progression, or if this raises questions regarding the definition of normal tissue as indicated by epigenetic changes (28).

Epigenetic changes affect CRC development by modulating the expression of various genes, such as tumour-suppressor and oncogenes (16, 18). Although the mechanisms are less well-understood, the idea of viral modification of the host epigenome is conceptually satisfying (29). This may involve repression of tumour-suppressor genes, such in the case of the Epstein-Barr virus and Kaposi's sarcoma-associated virus (29). Recently, we showed that differences exist between Swedish and Vietnamese patients with CRC regarding DNA methylation of the tumour suppressor adenomatous polyposis coli (*APC*) and insulin like growth factor binding protein 7 (*IGFBP7*) gene promoter region in cancerous and normal tissue (30).

A limitation of our study is that for the investigated Vietnamese patients (n=83), we had access to only 36 blood samples. In Sweden, the HMCV seroprevalence is about 70% and in Asian countries up to 100% (2, 4, 5-7). There are no comprehensive or documented general studies on HCMV seroprevalence in Vietnam. In this study, the Vietnamese patients had a significantly higher rate of HCMV seropositivity (100%) compared to Swedish patients (70.7%). In addition, we found that the Swedish HCMV-seropositive group had a rate of HCMV DNA-positivity of 21.9% in cancer tissue. This result is significantly lower than the 39.8% found in Vietnamese patients assuming a seroprevalence of 100%. However, the significant difference would remain, even if fewer than 100% of the Vietnamese patients were seropositive.

The present study does not claim to illustrate causality in colorectal carcinogenesis but to indicate that CRC tissue is significantly burdened with HCMV DNA and that this could also be linked to ethnicity and disseminated cancer.

In summary, our observations indicate that the rate of HCMV DNA-positivity differs significantly between cancerous and matched normal tissues. Furthermore, the rate tended to be higher in disseminated disease compared to localized disease in the Vietnamese cancer tissues. Thus, our investigation supports a possible role of CMV in CRC. Moreover, we noted differences between Swedish and Vietnamese patients, indicating a possible role of ethnicity. If the burden of HCMV

DNA in cancerous tissue affects cancer development and prognosis, the 5-year survival rate would be an interesting indicator to follow.

Acknowledgements

This work was supported by grants from Futurum the Academy of Healthcare, County Council of Jönköping, Sweden, the Foundation of Clinical Cancer Research, Jönköping, Sweden and the University College of Health Sciences, Jönköping Sweden. This work was also supported by a grant from KC.04.10/11-15 project of Ministry of Science and Technology, Vietnam. We thank Dr Tran Van Tuan at Department of Cytology and Pathology, National Cancer Hospital, Tam Hiep, Hanoi, Vietnam for having provided us with tissue from Vietnamese with colorectal cancer patients. We also thank Olaf Dienus for technical support.

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Received September 13, 2013
Revised October 18, 2013
Accepted October 21, 2013