Identification of human cytomegalovirus in tumour tissues of colorectal cancer and its association with the outcome of non-elderly patients

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Increasing evidence suggests that human cytomegalovirus (HCMV) plays an oncomodulatory role in human cancers. In colorectal cancer (CRC), presence of HCMV in tumours has been associated with a poor outcome in elderly patients. This study aimed to investigate the association between HCMV and the outcome of non-elderly patients with CRC. In tumour samples, HCMV DNA was detected by PCR. Viral transcript and protein were detected by in situ hybridization (ISH) and immunohistochemical staining (IHC), respectively. Clinical, pathological and survival data were compared between patients with HCMV-positive and -negative tumours. Quantitative reverse transcription PCR (qRT-PCR) was used to analyse the expression levels of cellular signals related to CRC progression and metastasis. Among 89 CRC non-elderly patients aged <65 years, HCMV was detected in 31 (34.8%) tumour samples by PCR. By ISH and IHC, viral transcript and protein specifically localized to the cytoplasm of neoplastic mucosal epithelium. Outcome analysis revealed a more favourable disease-free survival (DFS) rate in patients with HCMV-positive tumours (P<0.01), specifically in patients with stage III disease. In a multivariate Cox proportional-hazard model, tumoural presence of HCMV independently predicted a higher DFS rate (hazard ratio 0.22; 95% confidence interval 0.075–0.66, P<0.01). By qRT-PCR, the tumoural levels of interleukin-1 were relatively lower in samples positive for HCMV. The results suggest that HCMV may

One supplementary table is available with the online Supplementary Material.
INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality and morbidity worldwide. Although multiple treatment modalities including surgery, radiation and chemotherapy have been developed, the survival rate is low, except in cases of early detection (Haggar & Boushey, 2009). Multiple factors contribute to the prognosis of CRC. The tumour-node-metastasis (TNM) classification of the Union for International Cancer Control, which is based on anatomical and histological characteristics of tumours, is the traditional gold standard for outcome prediction. However, even patients with the same disease stage and treatment have different outcomes. Recently, the tumour microenvironment has been recognized as one of the major factors influencing cancer progression and metastasis (Peddareddigari et al., 2010). However, the underlying mechanisms associated with these microenvironmental changes remain unclear.

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus that infects a majority of the human population. HCMV causes life-threatening diseases in immunocompromised hosts. On the other hand, in healthy individuals, the primary asymptomatic infection is usually followed by lifelong latency. During latent infection, the virus is able to modulate multiple cellular functions and signal pathways through complex virus-host interactions, many of which are associated with oncomodulation (Michaelis et al., 2009). A number of human cancers are suggested to be associated with HCMV (Cobbs et al., 2002; Giraldo et al., 1980; Huang et al., 2002; Pacsa et al., 1975; Sanford et al., 1977). In CRC, controversy exists around the presence of HCMV in tumour tissue. Some earlier studies reported negative detection of HCMV in the tumour tissue of CRC (Akin-tola-Ogunremi et al., 2005; Boguszakova et al., 1988; Brichácek et al., 1980). However, more and more recent data have indicated that HCMV preferentially infects the tumour tissue of CRC (Cai et al., 2016; Dimberg et al., 2013; Li et al., 2015; Salyakina & Tsinoremas, 2013; Sole et al., 2015; Tafvizi & Fard, 2014; Taher et al., 2014). There is also a growing body of evidence suggesting that HCMV plays an oncomodulatory role and affects the disease course of human cancers. In human glioma, tumoural presence of HCMV was linked to oncongenesis and tumour progression (Cobbs et al., 2014). Treatment with the antiviral agent valganciclovir significantly improved patients’ survival (Söderberg-Nauclér et al., 2013; Stragiootto et al., 2013). We previously found that the nucleic acids of HCMV specifically localized in the neoplastic mucosal epithelium of CRC (Chen et al., 2012). Analysis of elderly CRC patients revealed that tumoural presence of HCMV was associated with shorter disease-free survival (DFS) and independently predicted tumour recurrence after surgery (Chen et al., 2014). In the present study, we aimed to clarify the association between tumoural presence of HCMV and the outcome of non-elderly patients with CRC.

RESULTS

Detection of the human cytomegalovirus of colorectal cancer in non-elderly patients

A total of 178 paired tumorous and adjacent non-neoplastic specimens were obtained from 89 CRC patients aged <65 years old. By PCR, HCMV was detected in 31 (34.8%) of the tumour samples and much less frequently in 7 (7.8%) of the adjacent non-neoplastic specimens (P<0.0001). By gene sequencing, the PCR products were proved to be specific for HCMV. The specific localization of HCMV in the tumour tissue of CRC was identified by in situ hybridization (ISH) and immunohistochemical staining (IHC) using HCMV-specific nucleic acid probe and HCMV-specific antibody, respectively. By ISH, the nucleic acids of HCMV were found in the cytoplasm of neoplastic mucosal epithelial cells of PCR-positive tumour specimens (Fig. 1). The extent of ISH staining was concordant with the intratumoural viral loads (Fig. 1b–d), while PCR-negative tumour samples showed no hybridization (Fig. 1e). No hybridization was detected in the stroma of submucosa (Fig. 1b–d). By IHC, the immunoreactivity against HCMV immediate early (IE) and early antigens was detected mainly throughout the neoplastic mucosal epithelium (Fig. 1i, j). Similar to the results of ISH, the immunoreactivity of HCMV in CRC was demonstrated in the cytoplasm of neoplastic cells, showing a clear difference from the nuclear immunoreactivity that is frequently seen in HCMV colitis (Fig. 1I).

Demographic characteristics, clinical features and pathological findings

Patients with HCMV-positive tumours were older than patients with HCMV-negative tumours (Table 1). The frequency of HCMV-positive tumours was lowest in patients of the youngest age quartile (mean age, 44.9±5.5 years; Fig. 2a). Patients with stage III disease more likely had HCMV-positive tumours than those with other stage cancers (Fig. 2b). The preoperative levels of tumour marker carcinoembryonic antigen (CEA) were not different between the two groups (P>0.3). HCMV presence was not associated with a different
grade of differentiation or chance of presence of pathological signs of early metastasis, including vascular emboli, lymphatic invasion and perineural invasion. The local extent of tumour (T) or chances of regional lymph node metastasis (N) and distant metastasis (M) did not differ between the two groups of patients. Significant intratumoural inflammation was more frequently observed in HCMV-positive tumours (11/31 vs 8/58, \( P < 0.031 \)).

**Human cytomegalovirus in tumour was associated with favourable disease-free survival in non-elderly patients with colorectal cancer**

The median follow-up duration was 32.9 and 3 months for patients with HCMV-negative tumours and 35.9 months for those with HCMV-positive tumours. The Kaplan–Meier analysis demonstrated the DFS curve of all 89 CRC patients aged <65 years according to the TNM stage (Fig. 3a). For all non-elderly patients, presence of HCMV-positive tumours had a more favourable DFS rate (\( P < 0.01 \), Fig. 3b). The DFS rates did not differ between stage II or III patients with HCMV-positive tumours and stage II patients with HCMV-negative tumours, while the presence of HCMV-negative tumours in stage III disease indicated an unfavourable DFS rate (Fig. 3c). In stage I or IV patients, the DFS rates did not differ regardless of presence or absence of HCMV in the tumour tissue (Fig. 3d). When the survival was analysed in different age groups, HCMV-associated survival difference was mainly observed in the 3rd (age 56.1–60.4 years, \( P < 0.1 \), Fig. 4c) and 4th age quartiles of patients (age 60.8–64.9 years, \( P < 0.05 \), Fig. 4d).

The univariate and multivariate Cox proportional-hazards models for 5 year cancer recurrence are displayed in Table 2. The age and preoperative CEA levels were transformed into quartiles. Among the risk factors analysed, tumoural presence of HCMV independently predicted a higher DFS rate (hazard ratio (HR) 0.22; 95% confidence interval (CI) 0.075–0.66, \( P < 0.01 \)), while regional lymph node involvement and distant metastasis were associated with more frequent disease recurrence (HR 6.44 and 3.97, 95% CI 2.14–19.40 and 1.79–8.79, \( P < 0.005 \) and \( < 0.005 \), respectively).

**Alteration of cellular signals associated with tumoural presence of human cytomegalovirus**

Transcription levels of several cellular signals associated with invasiveness and metastasis of CRC were analysed in tumour samples from non-elderly patients. Relative levels in tumour as well as the ratio of levels in tumour versus normal tissues were compared between HCMV-positive and -negative samples, including cyclooxygenase (COX)2, matrix metalloproteinases (MMP)1, tissue inhibitor of metalloproteinases (TIMP)1, tumour necrosis factor (TNF)-\( \alpha \) and IL-1. Among the factors compared, the tumour levels of IL-1 and the tumour versus normal levels of IL-1 and COX2 were relatively lower in samples with HCMV infection (Fig. 5).
We recently demonstrated that tumoural presence of HCMV indicated a poor outcome in elderly patients (Chen et al., 2014). In the present work, surprisingly, we found that tumoural presence of HCMV correlated with a favourable survival outcome in non-elderly CRC patients. In a Cox regression model, tumoural presence of HCMV independently predicted DFS in non-elderly CRC patients. Considering the results of the two studies together, HCMV seems to confer a dual oncomodulatory effect on CRC, depending on patient age.

The preferential localization of HCMV in tumorous epithelium of CRC was demonstrated by ISH and IHC. In contrast to the nuclear distribution in non-permissive cells, the results of ISH with the probe targeting the \( \beta2.7 \) transcript of HCMV showed an intracytoplasmic hybridization in tumour cells, featuring a productive infection (Wu et al., 1992). Nevertheless, the similar cytoplasmic staining in IHC was contrary to the nuclear IE immunoreactivity found in productive HCMV infection such as colitis. Cytoplasmic distribution of HCMV IE proteins has been noted late after infection (Tsutsui & Yamazaki, 1991). Whether such

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**Table 1.** Demographic characteristics and underlying diseases in patients aged <65 years

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HCMV detected [n=31]</th>
<th>HCMV not detected [n=58]</th>
<th>( P )</th>
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</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>14 (45.2)</td>
<td>29 (50.0)</td>
<td>0.82</td>
</tr>
<tr>
<td>Age (mean years±SD)</td>
<td>56.8±5.2</td>
<td>53.8±8.0</td>
<td>0.037</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (6.5)</td>
<td>8 (13.8)</td>
<td>0.48</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5 (16.1)</td>
<td>19 (32.4)</td>
<td>0.13</td>
</tr>
<tr>
<td>Heart diseases</td>
<td>4 (12.9)</td>
<td>4 (6.9)</td>
<td>0.44</td>
</tr>
<tr>
<td>Renal diseases</td>
<td>1 (3.2)</td>
<td>1 (1.7)</td>
<td>1.00</td>
</tr>
<tr>
<td>Liver diseases</td>
<td>1 (3.2)</td>
<td>1 (1.7)</td>
<td>1.00</td>
</tr>
<tr>
<td>Central nervous system diseases</td>
<td>5 (11.6)</td>
<td>7 (5.8)</td>
<td>0.46</td>
</tr>
<tr>
<td>History of polyp or CRC</td>
<td>0 (0.0)</td>
<td>2 (3.4)</td>
<td>0.54</td>
</tr>
<tr>
<td>History of other malignancy</td>
<td>3 (9.7)</td>
<td>3 (5.2)</td>
<td>0.66</td>
</tr>
<tr>
<td>Curative surgery</td>
<td>30 (96.8)</td>
<td>48 (84.2)</td>
<td>0.091</td>
</tr>
<tr>
<td>Ascending colon cancer</td>
<td>11 (35.5)</td>
<td>16 (27.6)</td>
<td>0.48</td>
</tr>
<tr>
<td>Rectal cancer</td>
<td>11 (35.5)</td>
<td>16 (28.1)</td>
<td>0.63</td>
</tr>
<tr>
<td>Poorly differentiated tumour</td>
<td>2 (6.5)</td>
<td>2 (3.5)</td>
<td>0.61</td>
</tr>
<tr>
<td>Prominent inflammation in tumour</td>
<td>11 (35.5)</td>
<td>8 (14.3)</td>
<td>0.031</td>
</tr>
<tr>
<td>Pathological signs of early metastasis: VELIPI*</td>
<td>11 (35.5)</td>
<td>14 (25.0)</td>
<td>0.33</td>
</tr>
<tr>
<td>Advanced local invasion (T4)</td>
<td>4 (12.9)</td>
<td>7 (12.1)</td>
<td>1.00</td>
</tr>
<tr>
<td>Regional lymph node involvement</td>
<td>18 (58.1)</td>
<td>28 (48.3)</td>
<td>0.51</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>5 (16.1)</td>
<td>15 (25.9)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*VELIPI represents pathological features of early metastatic invasion, including vascular emboli, lymphatic invasion, and perineural invasion, alone or in combination.

**DISCUSSION**

We recently demonstrated that tumoural presence of HCMV indicated a poor outcome in elderly patients (Chen et al., 2014). In the present work, surprisingly, we found that tumoural presence of HCMV correlated with a favourable survival outcome in non-elderly CRC patients. In a Cox regression model, tumoural presence of HCMV independently predicted DFS in non-elderly CRC patients. Considering the results of the two studies together, HCMV seems to confer a dual oncomodulatory effect on CRC, depending on patient age.

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**Fig. 2.** Distribution of HCMV-positive and -negative tumours in non-elderly patients with CRC according to (a) tumour stages and (b) age quartiles.
findings represented a different viral physiology of HCMV in tumour cells requires further investigation.

Based on the results of this study and our previous work that compared the viral loads in tumour and normal specimens of CRC (Chen et al., 2012), we speculate that HCMV directly infects, or reactivates within, the neoplastic epithelium of CRC. Virus replication possibly correlated with changes in the tumour microenvironment, since the serologic and viraemic status of HCMV is not associated with viral replication and transcription in tumour tissues (Chen et al., 2016). Furthermore, since the ISH probe targeted the most abundantly transcribed viral gene during permissive infection (McSharry et al., 2003; Wu et al., 1992), the virus is probably not 'dormant' in tumours of CRC. HCMV may have been carried by the myeloid progenitor cells which it latently infected and reactivated in the neoplastic cells with active viral replication and viral gene expression.

The finding that tumoural presence of HCMV is associated with a favourable outcome in non-elderly patients was surprising, but not unfathomable, considering the pathophysiology of HCMV infection. Increasing evidence suggests that HCMV has a dual or even contradictory effect on the host, depending on the age. In the elderly, chronic latent HCMV infection increases the mortality rate, which could be attributed to HCMV-induced immunosenescence with decreased immunity to microbes and cancers (Pawelec et al., 2010; Sansoni et al., 2014; Savva et al., 2013). In the non-elderly, on the other hand, chronic HCMV infection may actually be beneficial in that it augments the T-cell immunity and has been associated with a reduced cancer risk in transplant patients (Couzi et al., 2010; Furman et al., 2015; Pera et al., 2014). In this study, significant intra-tumoural inflammatory responses were more frequently observed in HCMV-positive tumours, suggesting that viral replication may

Fig. 3. Kaplan–Meier curves of DFS in non-elderly patients with CRC. (a) All patients stratified according to tumour stages. (b) All patients stratified according to the tumoural presence or absence of HCMV. (c) Stage II and III patients, and (d) stage I and IV patients stratified according to the tumoural presence or absence of HCMV. In (d), the survival line of HCMV(+) stage I overlaps that of HCMV(−) stage I. NS, not significant
activate the immune response in the tumour microenvironment and contribute to a favourable prognosis (Naito et al., 1998). In addition, the levels of IL-1 were lower in HCMV-positive tumours. IL-1 is a pleiotropic cytokine known to promote tumour growth, angiogenesis and metastasis (Lewis et al., 2006), while COX2 plays a pivotal role in the oncogenesis and progression of CRC (Greenough et al., 2009; Tsujii et al., 1997). Whether HCMV influences the outcome of CRC through modulating this pathway in the tumour microenvironment should be further investigated.

Based on these results, we speculate that HCMV exerts a dual oncomodulatory effect on CRC in an age-dependent manner. In non-elderly patients, HCMV infection of the cancer cells may attenuate cellular signals of cell growth and metastasis, and activate the anti-cancer immune response in the tumour microenvironment. In the elderly, on the contrary, such beneficial effects were absent, while other detrimental effects prevailed. Possible mechanisms include HCMV-induced local immunosenescence in the tumour microenvironment or an imbalanced cellular immune response, as indicated by our previous study (Chen et al., 2014). The results of our

**Table 2.** Cox proportional-hazards models for prediction of 5 year mortality in patients aged <65 years

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Cox model</th>
<th>Multivariate Cox model</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCMV in tumour (yes/no)</td>
<td>HR (95 % CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>0.32 (0.13–0.79)</td>
<td>0.013</td>
</tr>
<tr>
<td>Preoperative CEA, quartile</td>
<td>1.80 (1.24–2.62)</td>
<td>0.002</td>
</tr>
<tr>
<td>VELIPI* (yes/no)</td>
<td>4.29 (2.04–9.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TNM stage (I–IV)</td>
<td>4.56 (2.66–7.80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regional lymph node involvement (yes/no)</td>
<td>7.92 (2.77–22.63)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distant metastasis (yes/no)</td>
<td>7.80 (3.84–15.84)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NA, Not applicable.

*VELIPI represents pathological features of early metastatic invasion, including vascular emboli, lymphatic invasion, and perineural invasion, alone or in combination.
previous and present studies also indicated a noteworthy issue: when studying the oncomodulatory role of HCMV, the non-elderly and elderly hosts should be analysed separately as HCMV may have a different influence on these two populations.

**METHODS**

**Study specimens**

The study was approved by the Institutional Review Board of Taipei Veterans General Hospital (VGHTPE). The tumour specimens were
randomly retrieved from the bank of residual surgical tissues at the Division of Colorectal Surgery, VGH-TPE. These samples were collected from CRC patients who underwent primary resection between 2000 and 2012. There were no eligibility criteria for gender, ethnicity and disease staging.

Detection of human cytomegalovirus in colorectal cancer

**PCR and gene sequencing.** To detect HCMV in CRC, PCR was carried out using primers targeting viral genes UL55, UL73 and UL144, as previously described (Chen et al., 2012). The reactions were carried out with an initial temperature of 95°C for 5 min; 35 cycles of 95°C for 1.5 min, 55°C for 2 min and 72°C for 1 min; and an additional 10 min at 72°C in the last cycle. Subsequently, gel electrophoresis was conducted for the PCR products, using a 1.5% agarose gel; the gel was stained and photographed under ultraviolet light. Presence of HCMV was defined as a positive PCR result for any of the abovementioned viral genes tested. The amplification products of viral genes were gel-eluted (EasyPure PCR Gel Extraction kit; Bioman Scientific) and subjected to sequencing (Mission Biotech).

**In situ hybridization.** ISH of CRC specimens was performed using methods previously described (Chen et al., 2012). Briefly, paraffin-embedded pathological samples were deparaffinized by heating in a 65°C oven for 15 min and then washed twice in xylene. Sections were serially rehydrated in 100, 95 and 75% ethanol, and postfixed with neutral buffered formalin. After pepsin treatment at 37°C for 8 min and initial denaturing at 90°C for 25 min, hybridization was carried out with the probes at 42°C for 17 h and then the sections were washed with 1× washing buffer and distilled water. For HCMV detection, a biotinylated 21-base oligonucleotide probe specific for the β2.7 transcript of HCMV was used (ZytoVision). A biotinylated probe specific for human Alu sequences was adopted as a positive control. A set of biotin-marked random sequence oligonucleotides with GC contents ranging from 40 to 70% was used as a negative control. The probes were detected with an alkaline phosphatase detection system using nitro-blue tetrazolium as a chromogen. Slides were visualized under the microscope after counterstaining with nuclear fast red.

**Immunohistochemical staining.** Deparaffinized 5μm sections were subjected to antigen retrieval, using Tris-EDTA buffer pH 9.0 for 20 min at 95°C and 30 min incubation at room temperature. Slides were treated with H2O2 for 10 min to block non-specific cellular peroxidase. After blocking with 2% goat serum for 20 min, sections were incubated with prediluted mouse monoclonal anti-HCMV antibody specific for HCMV IE and early antigens (clone DDG9 & CCH2; Abcam) and a secondary antibody conjugated with horseradish peroxidase-labelled polymer (EnVision Dual Link System-HRP; Dako). Sections were then incubated with the chromogen diaminobenzidine for 15 min and visualized under the microscope.

Collection of clinical and pathological data

Demographic, clinical and pathological data were collected as previously described (Chen et al., 2014). For tumour staging, the TNM classification was used (Sobin et al., 2009). The observation time was the interval between the diagnosis and the last follow-up or death. Data were censored at the last follow-up for patients who had not shown relapse and those who had died. DFS was defined as the period from the date of surgery to the date of confirmed tumour relapse for patients with relapse of unresectable tumours, or from the date of surgery to the date of the last follow-up for patients without detectable tumour.

**Quantitative reverse transcription PCR (qRT-PCR)**

Total RNA was extracted from CRC specimens with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Reverse transcription was carried out using 2 ml 10× reaction buffer, 2 ml 10× random hexamers, 0.8 ml 100 mM dNTP mix, 1 ml MultiScribe reverse transcriptase (Life Technology), 1 ml RNase Inhibitor (Applied Biosystems) and 2 mg sample RNA and diethylpyrocarbonate (DEPC) H2O in a total volume of 10 ml. Reverse transcription was carried out with the following protocol: 25°C for 10 min, 37°C for 120 min; the reaction mixture was heat inactivated at 85°C for 5 min and then chilled on ice. The cDNA was stored at −20°C until use. Quantitative PCR reaction was carried out by mixing 10 μl 2× Smart Quant Green Mix (Protech Technology), 2 μl cDNA, 1 μl forward and reverse primers (500 nM each primer) and 6 μl H2O. Thermal cycling conditions were similar for all qRT-PCR reactions, with 3 min of initial denaturation and enzyme activation at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C and 1 min of final reaction at 95°C. For each reaction, a melt curve was generated by heating from 55°C to 95°C, with 0.5°C incremental steps of 10 s. The sequences of primers are listed in Table S1 (available in the online Supplementary Material). Expression levels of genes were quantified on the basis of intercalation of SYBR Green on an ABI 7000 Real-Time PCR system (Life Technologies). Data normalization and analysis were accomplished using the comparative cycle threshold (Ct) method. Each replicate Ct was normalized to the Ct of the human housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The fold change in genes relative to the GAPDH endogenous control was calculated as 2ΔΔCt.

Statistical analysis

Categorical variables were compared by using the chi-squared test with Fisher’s exact test. Continuous variables were compared by using the t-test for data that followed the normal distribution and the Mann–Whitney U-test for data that did not follow the normal distribution. Survival was evaluated by using the Kaplan–Meier method, and significant differences in survival curves were evaluated with the Mantel–Cox log rank test. A Cox proportional-hazards regression model with forward stepwise selection procedure was used to identify risk factors for 5-year mortality. Values of P<0.1 in the univariate analysis were required for a variable to be entered into the multivariate model. To reduce the right skewness in the multivariate analysis, patient age and preoperative CEA levels were transformed into quartiles. A value of P<0.05 indicated a significant difference. All analyses were carried out using SPSS18.0 for Windows (IBM-SPSS).

**ACKNOWLEDGEMENTS**

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**REFERENCES**

after kidney transplantation.

Garrigue, I., Hawchar, O., Siberchicot, F. & other authors (2010).


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