Detection of Human JC Polyomavirus DNA in Patients with Solid Tumors by Semi-Nested Polymerase Chain Reaction

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Abstract: Background: JCV is a DNA polyomavirus identified in human cancers. In Egypt, very limited data about presence of JC polyomavirus in immunocompromised cancer patients. Here, we investigated JCV infection and viral DNA load in Egyptian patients with different tumors and its possible correlation with outcome of the disease. **Patients and Methods:** Fifty blood samples were collected from cancer patients with different solid tumors, and 20 blood samples were collected from apparently healthy individuals as a control group. All samples were subjected to detection of JC polyomavirus by seminested PCR (snPCR) assay using primers targeting large T antigen and real time PCR to measure viral load. **Results:** The sensitivity level of snPCR assay was 100 copies/ul for JC polyomavirus, while the sensitivity level of quantitative PCR assay was found to be 10 copies/ul. Our snPCR assay was highly specific. JC polyomovirus DNA could be detected in 6 of 51 (11.8%) patients with solid tumors, but none of control was positive for JC polyomavirus DNA. Median viral load was found to be 200 copies/ul of serum. **Conclusion:** Elevation of JC polyomaviruses copies in patients with different human cancers suggesting its correlation with pathogenesis of disease, but further analysis is required on a larger scale to confirm such findings.
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Key words: JC polyomavirus infection, snPCR assay, cancer patients

1. Introduction

John Cunningham virus (JCV), is a member of the polyomaviridae family, small non enveloped virus with circular double stranded DNA genome, ranging in size from 45 to 55 nm. JC polyomavirus infects humans ubiquitously, about 70% to 80% of the adult population having JC virus-specific antibodies. Several studies demonstrated that JC virus infection takes place during early childhood and remains subclinical. After primary infection, JC virus can be found in the kidneys, B lymphocytes, and gut mucosa [1].

Initial infection is thought to occur in the tonsils [2] or more likely the gastrointestinal (GI) tract [3]. The virus then remains latent in the GI tract [4] and tubular epithelial cells of the kidneys [5]. Classically, pathogenesis of JCV has been limited to immunosuppressed patients, such as in acquired immunodeficiency syndrome and organ transplantation. In the immunosuppressed setting, reactivation of JCV occurs and can induce fatal demyelinating disease progressive multifocal leukoencephalopathy (PML) as well as polyomavirus associated nephropathy (PVAN) [6,7]. More recently, there has been a mounting evidence for a potential role of JCV in human cancers in the absence of immunosuppression or PML. JCV DNA sequences and its oncogenic T-antigen expression have been demonstrated in a variety of human cancers, including brain, [8] gastric [9,10] esophageal [11] and colon cancer [12-15].

Since 35 to 90% of healthy adult are seropositive for polyomaviruses (PoV) and JCV, serological assays are rarely used for diagnosing ongoing PoV associated diseases. Instead, polyomavirus DNA loads in urine and blood have become pivotal laboratory assays in the management of immunocompetent patients [16]. Several studies have demonstrated presence of JCV DNA or antigens in the peripheral blood (PB) or serum. Viral components detected in the blood have historically been presumed to have originated from metastasized cancer cells or from virus containing cellular debris shed from a local infection site [16,17].

There are very limited data about presence of JC polyomavirus infection among Egyptian cancer patients. Therefore we aimed at establishing sensitive and specific seminested version of PCR (snPCR) assay for detection and quantification viral load of JC polyomavirus and investigating its impact on outcome of disease.

2. Patients and Methods:

Study population

The current study composed of 51 cancer patients with solid tumors diagnosed and treated at Medical Oncology Department, National Cancer Institute- Cairo university (NCI) in the period between January 2010 till November 2011.

All patients fulfilled the inclusion criteria concerning age (Age ≥18 years), and treatment (no previous treatment nor antiviral treatment).

All patients were subjected to pretreatment assessment including: Full history, physical examination and baseline evaluation of the disease by blood analysis

(CBC, LFTs, KFTs), histological diagnosis, radiological assessment to determine the disease stage using AJCC-UICC 2010.

Twenty apparently healthy individuals age and sex matched were included as control group, they were taken from blood donors at NCI. The Institutional Review board (IRB) of the NCI approved the protocol. Informed written consent was obtained from all patients and individuals enrolled in the study.

Methods

Specimen collection

Serum samples were obtained from each patient and control and stored in aliquots at -20^{o}C until processed.

Molecular detection

1- Nucleic acid extraction: DNA was extracted using QIA amp viral RNA extraction kit (Qiagen,Valencia, USA). The extraction was done according to the manufacturer's instructions. The amount of viral DNA was measured by spectrophtometry using a Nano-Drop 2000 spectrophotometer (Thermo Scientific/US, Canada) and 100ng of DNA template was used in the PCR assays.

DNA extracts were placed on ice and were used immediately for PCR, then stored at -80^{o}C until further analysis.

Samples were subjected to semi-nested version of PCR targeting large T genomic region of JC polyomavirus (**Figure 1**).

2- Amplification of PoV DNA by snPCR assay

Amplification of JC polyomavirus was carried **out** in**to** 2 steps; the first amplification, JC virus was amplified using outer primers between 3978 to 4131 nucleotide position of JC polyomavirus genome. The amplification was done in a final volume of 25 µl of 5-Prime MasterMix (Berlin, Germany) containing 0.2 µM of each primer (BKJC1, BKJC2) according to previously published protocol [18]. JC viral DNA concentration was measured by spectrophtometry using a Nano-Drop 2000 spectrophotometer (Thermo Scientific/US, Canada) and 100ng of DNA template was used in the PCR assays. JC polyomavirus VR-1583 (ATCC-USA) was supplied as a gift by WHO Collaborating Center for Acquired Immunodeficiency Syndrome (AIDS) and WHO Collaborating Center for Virus Reference and Research for the Eastern Mediterranean Region (EMR), Virology Unit, Department of Microbiology, Faculty of Medicine, Kuwait University and was extracted by the same extraction procedure for samples. JC polyomavirus positive and negative (water) controls were run in each PCR assay.

In the second amplification (semi-nested) the primers targeting position from 4254 to 4325 of the JCV genome were designed using primer 3 software. Positive samples for JCV were used as template control as it was not possible to obtain their ATCC reference sample**(Plz omit this sentence).**

3. Detection of amplified product by agarose gel electrophoresis:

Fifteen microliter of the PCR product was subjected to electrophoresis on a 2.5% agarose gel (Sigma) in Tris-Acetate buffer (TAE 1X) pH 8.2, stained with 0.5µg /ml ethidium bromide and examined under UV transillumination and photographed. Product sizes were estimated by comparison with 100bp DNA ladder (Amersham, UK). Amplified fragment was 71 bp for JCV (**Figure 1**).

4-Sensitivity and specificity of snPCR assay

The snPCR assay was subjected to sensitivity assay by making serial dilutions of positive control from 10 up to 10^{-7}. Specificity assay was performed against most of viruses run in virology&Immunology unit to exclude contamination.

5-Detection of PoV by quantitative Real Time PCR

Detection and quantification of PoV viral load was done by real time detection system (Applied Biosystems StepOne TM Real Time PCR system Thermal Cycling Block, Singaphore). The amplification of the BK-JC viruses was done using the common outer primers. The reaction mixture contained 12.5µl of Maxima SYBR Green/ROX qPCR master mix (2X) (Fermentas, California, USA), 10µM of each of outer primers, and 100ng of extracted DNA in a final volume of 25µl distilled water. The BK/JC cycle protocol consisted of 4 stages: stage 1 (held 95.0^{o}C for 9 min.); stage 2 (3 temperature cycles, repeated 40 times, at 95.0^{o}C for 1min; 55.0^{o}C for 1min,; and a final step of 72.0^{o}C for 1min); stage 3 (melting curve 95.0^{o}C for 15sec.; 60^{o}C for 1 min , and 95^{o}C for 15 sec); and stage 4 (cooling at 4^{o}C for infinity). During the post amplification melting temperature Tm analysis, the PCR products were subjected to a temperature increase of up to 85^{o}C, resulting in a progressive decrease of fluorescence. In this way it was possible to distinguish nonspecific PCR products from the BK and JC-specific product sequences.

Standard curve

Standard curve was constructed using 10-fold serial dilutions (10 to 10^6 copies/ml) of polyoma JC polyomavirus standard control with known concentration of 300 fg of extracted DNA that was equivalent to one copy of viral DNA.

Controls

A total of 5 Controls were used in each assay as follows: AC (negative amplification control), NC (negative sample for BK/JC viruses), BK virus standard, positive sample for BK virus and JC positive control of known concentration (100ng/ul). The AC consisted of all reagents used in the reaction mixture but with sterile water added in place of DNA template.

Sensitivity and Specificity of real time PCR

Sensitivity was performed by testing our polyomaviruses primers against JC virus previously prepared in serial 10 fold dilutions, while specificity was performed by testing our primers used for detection of polyomaviruses against samples positive for parvovirus, CMV, HHV6, EBV and HBV which are available in our laboratory.

Statistical analysis.

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. Survival analysis was done using Kaplan-Meier method *P*-value < 0.05 was considered significant.

3.Results

This prospective cross sectional study composed of 51 solid tumor cancer patients, 18 had breast cancer, 8 had rectal cancer, 7 liver cancer, 3 brain cancer, 3 ovarian cancer, 2 cervical carcinoma, 2 plural mesothelioma, 2 testicular carcinoma, 1 laryngeal carcinoma, 1 bladder cancer, 1 non-small cell lung carcinoma (NSCLC), 1 penis sarcoma and 2 synovial sarcoma. Age and sex distribution, some clinical and hematological data of cancer patients are presented in **table1**.

Disease characteristics

Status of disease was divided according to response criteria [19] into complete remission, partial remission and relapse and they are defined as follows:

Complete remission (CR): Presence of <5% blasts as indicated by morphologic evaluation of bone marrow; no evidence of Auer rods, leukemia-specific karyotype, circulating blasts, or extramedullary disease; and evidence of hematopoietic recovery (ANC> 300/ul and rising; platelet count 30, 000/ul and rising).

Partial remission (PR): Bone marrow is generating normal hematopoietic cells and contains 5-15% blast cells by morphology and no evidence of extramedullary disease after the 1st course of induction therapy.

Relapse (R): Morphologic relapse after CR is defined as reappearance of leukemic blasts in the peripheral blood or > 5% blasts in the bone marrow not attributable to any other cause [20].

Our results showed that in solid tumors: thirty sex of 51 (70.5%) patients showed (CR+PR), 15 (29.5%) had relapsed disease **table (1)**.

Sensitivity and specificity of qualitative PCR assay for detection of polyomaviruses DNA:

Amplification of polyomaviruses DNA was performed using 1st round PCR assay using two sets of primers targeting large T genomic region of polyomaviruses genome (BKV&JCV), the amplicon size of the first amplification step was at 175bp as shown in **Figure (1)**:

The seminested PCR (snPCR) amplification of JC polyomavirus gave fragment length at 71 bp was shown in **Figure** (**2**).

***Sensitivity and specificity of PCR assay*:**

The sensitivity level of the assay was detected to be approximately 100 copies/ µl for polyomavirus viruses **Figure (3)**.

The PCR was found to be highly specific for polyomaviruses (BK&JC) as none of the other tested viruses were amplified (EBV, CMV, HSV1, HSV2, VZV, HBV)

Sensitivity and specificity of real time PCR assay for polyomaviruses (PoV) DNA.

The polyomaviruses standard curve was constructed in the range from 10 to 10^6 copies/ml. Post amplification melting curve analysis was carried out to distinguish polyomavirus specific sequences. Peak melting temperatures were found to occur at 75=1ºC. The sensitivity of the assay was found to be 10 copies/reaction which represents lowest standard dilution could be detected. Also, Quantitative PCR assay was found to be highly specific for PoV as none of the tested viruses was detected by real time PCR (**Figures 4,5**).

Presence of JC polyomavirus DNA infection in cancer patients

Polyomaviruses DNA were detected in 28/51(55%) of solid tumor patients, but were not detected in the control individuals (0%) by first round PCR assay. Seminested version of PCR assay that was used to identify type of polyomaviruses (BK or JC) showed that 13 of 51 (25.5%) were positive for BK polyomavirus but only 6(11.8%) were positive for JC virus infection (**Table 2**). Our results showed that median level of viral load was 200/ul of serum and ranged from 10-85x10^6/ul of serum (Table2).

Moreover, when evaluating sensitivity of our snPCR, we found that out of 6 JCV positive patients, 4 cases were negative by 1st round PCR but could be detected by snPCR assay (*P*=0.481) (**Tables 3a, 3b**). One cancer patient out of total 28 positive patients who was negative by real time PCR could be detected by snPCR assay. On the other hand, 7 cases were positive by quantitative PCR were negative by 2nd round PCR (p= 0.774). All results are presented in **tables 3a, b**.

Presence of JC polyomavirus DNA and its association with hematological parameters in cancer patients

We found that there was no significant difference between patients with JC positive and those with negative polyomaviruses within normal and abnormal levels of biochemical parameters. But we found that presence of JC polyomavirus was significantly associated with leukopenia (*P*=0.025).

JC polyomavirus DNA and status of disease

Regarding status of disease, our results showed that 6 out of 14 (42.9%)of relapsed patients were positive for polyomavirus DNA, one of them was positive for JC polyomavirus. Five of 11 (45%) of dead patients were positive for polyomaviruses DNA, one of them was infected with JCV.

Polyomavirus infection and overall survival:

Our results showed that age was the only parameter affected overall survival in patients with solid tumors (*P*=0.027) (**Table 4, Figure 6**)

Table (1): Clinical and hematological, characteristics of the studied cancer patients

	Patient (n=51)
Age (Y) Median(Range)	49.0(22-77)
Sex	
M: n(%)	18(35.3)
F: n(%)	33(64.7)
M:F ratio	0.6:1
LFT Median (range)	
AST (IU/L)	22.5(12-62)
ALT (IU/L)	21.5(8-65)
T.Bil (mg/dl)	0.50(0.2-3.7)
KFT Median (range)	
Createnin(mg/dl)	0.80(0.4-3.1)
CBC Median (range)	
Hb (g/dl)	12.0 (7.3-15.4)
TlC (x10^9/l)	6.8 (1.9-18.6)
ANC (x10^9/l)	59.0 (10-91)
Lym (x10^9/l)	32.0 (4.8-80)
Mono(x10^9/l)	6.0 (2.0-28.5)
Plt(x10^9/l)	289.5 (0-679)
Status of disease	
CR: n(%)	30(58.8)
PR: n(%)	6(11.7)
R: n(%)	15(29.5)

LFT: Liver function test, AST: Aspartate aminotransferase, ALT: Alanin Aminotransferase, T. Bil: Total bilirubin, KFT: Kidney function test, CBC: Complete blood picture, Hb: Hemoglobin concentration, TLC: Total leukocytic count, Lym: total lymphocyte count, CR: complete remission, PR: partial remission, R: relapse.

Normal values:

AST(up to 42 U/L), ALT(up to 45 U/L), Total bilirubin (0.2-1.2 mg/dl), Createnin (0.5-1.5 mg/dl), Hb conc.(13.0-17.0 g/dl) TLC(4-11)x10^3 ccm, ANC(40 -70%), Lym(20-45%).

Table 2. Distribution of polyomaviruses (PoV) as detected by snPCR assay

Polyomavirus results (PoV)	Solid tumors n=(51)
PoV, n (%)	17(33)
BK, n (%)	13(25.5)
JC, n (%)	6(11.8)
Total positives, n(%)	28(55)
PoV load, median (range)	200 (10-85x10^6)

PoV: Polyomavirus, BK: BK-virus, JC: John Cunningham virus

Table (3a): Qualitative and quantitative screening of JCV in cancer patients:

Polyomaviruses (PoV) results	**Solid tumors (a) n=(51) Pos. = 28 (55%)**
PoV qualitative 1st round n (%)	17/28(61)
PoV qualitative 2nd round JCV n (%)	6/28 (21)
PoV quantitative pos. n (%)	23/28(82)
Viral load Median (range)	200 (10-85x10^6)
Positive JCV by real time PCR, n (%)	5/23 (22)

p-value > 0.05 not significant, p-value < 0.05 significant

PoV: Polyomavirus, JCV: John Cunningham virus

Table (3b): P values of Qualitative and quantitative PCR assays in all studied cancer groups

PCR assay	**P value**
Qualitative PCR assay 1st PCR vs. Qualitative 2nd round PCR 2nd PCR vs. Quantitative	 0.481 0.774
Quantitative PCR assay 1st PCR vs 2nd PCR	 0.027

p-value>0.05 is not significant;
p-value < 0.05 is significant.

Table (4): Overall survival in Cancer patients.

	Cumulative survival at 1 years (%)	Cumulative events	Censored	*P*-value
Sex				
Male n=18	70.5%	5	13	0.32
Female n=33	80.2%	6	27	
Age				
<50 N=28	87.2%	3	25	**0.027**
≥50 N=23	63.7%	8	15	
Total pos n=28	79.9%	5	23	0.51
Total neg. n=23	72.6%	6	17	
PoV				
Pos n=17	87.8%	2	15	0.26
Neg n=34	71.6%	9	25	
JCV				
Pos n=6	83.3%	1	5	0.76
Neg n=45	75.7%	10	35	
TLC				
<4 n=6	66.7%	2	4	0.67
>4 n=45	79.6%	9	36	
ANC				
<1500 n=1	100%	0	1	-
>1500 n=50	76.2%	11	39	

PoV: Polyomavirus; JCV:John Cunningham virus; TLC: Total leukocytic count;ANC, Absolute neurophilic count

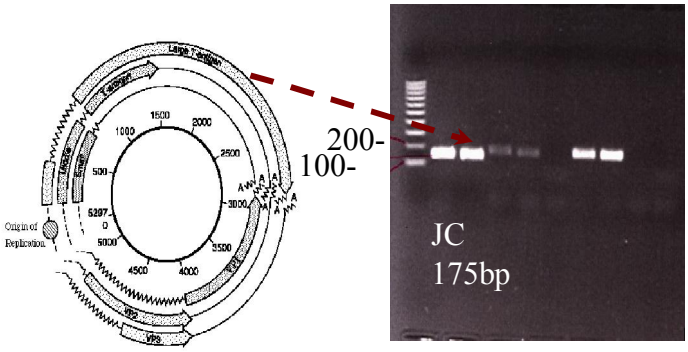


Figure (1): **PCR amplified T-region of polyoma JC virus at 175bp.**
Ethidium bromide-stained gel electrophoresis of polyomaviruses PCR products showing lane1=100bp ladder; lane 4,5= positive JCV; lane6= negative control; lane 8= positive sample for JC.

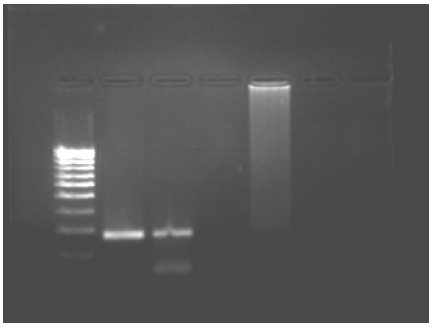
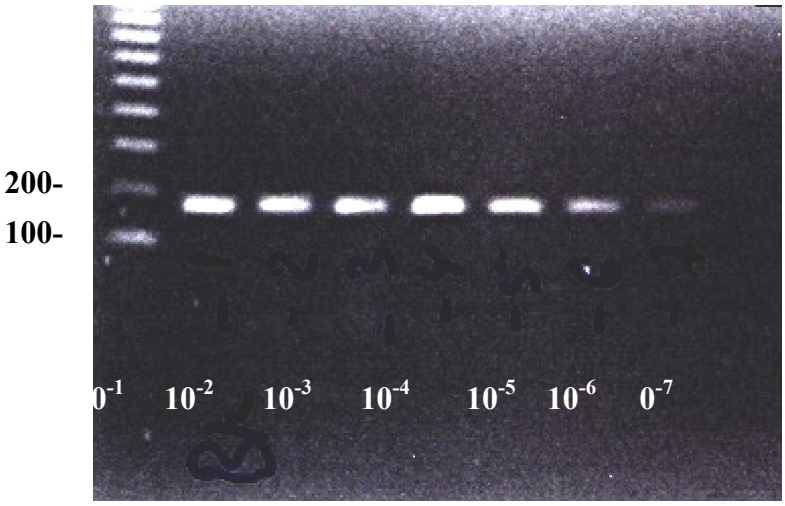


Figure (2): **Amplified fragment of polyomavirus JC DNA at 71bp**
Ethidium bromide-stained gel electrophoresis of polyoma JCV PCR product at **71** bp**.** lane 1=50 bp ladder; lanes 2,3= positive JCV; lane 4= Negative control**;** lane 5=Negative sample for JCV.



Figure(3): Sensitivity of qualitative PCR assay for detection of polyomavirus DNA

Ethidium bromide-stained gel electrophoresis of polyomaviruses PCR products showing serial dilution of PoV positive at 175 bp. lane 1=100 bp ladder; lane 2=10^{-1} positive PoV; lane 3=10^{-2} positive PoV; lane 4=10^{-3} positive PoV; lane 5=10^{-4} positive PoV; lane 6= 10^{-5} positive PoV; lane 7=10^{-6} positive PoV; lane 8=10^{-7} positive PoV; lane 9=Negative control.

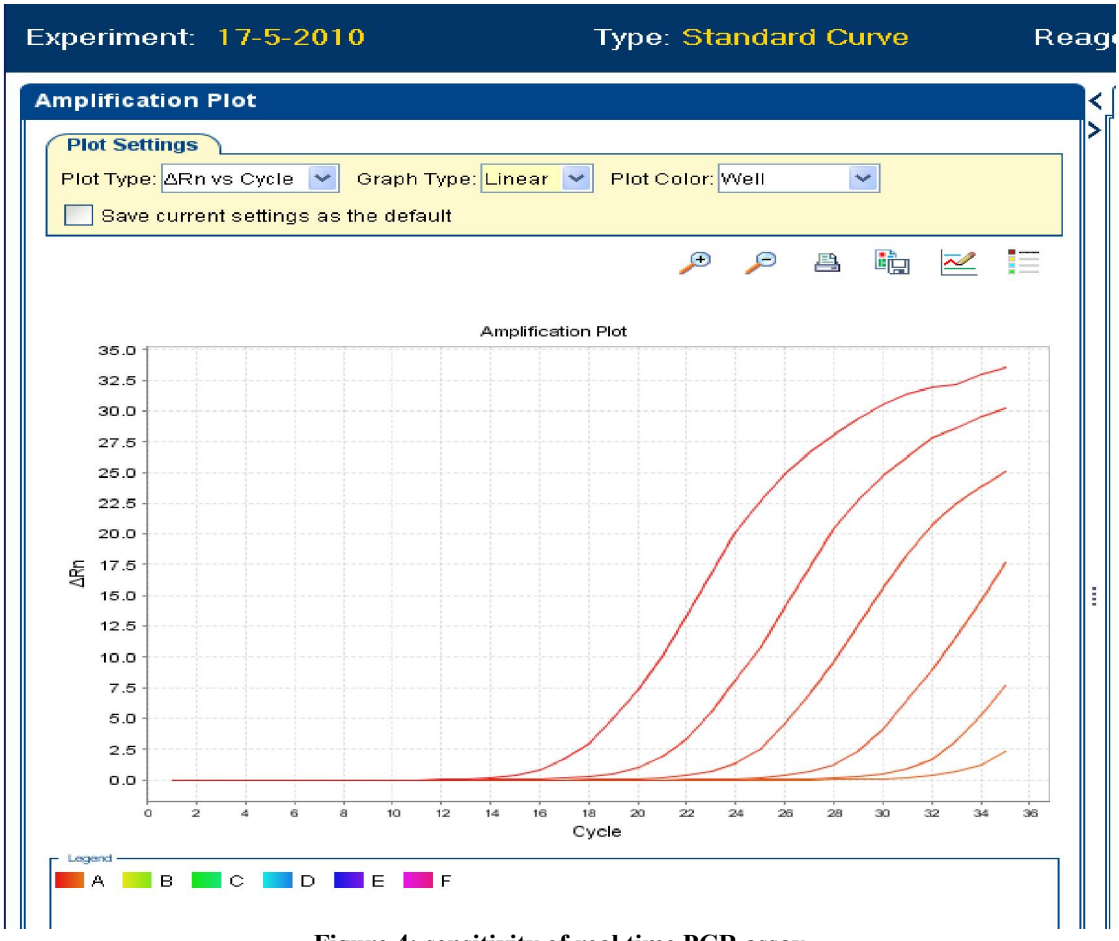


Figure 4: sensitivity of real time PCR assay

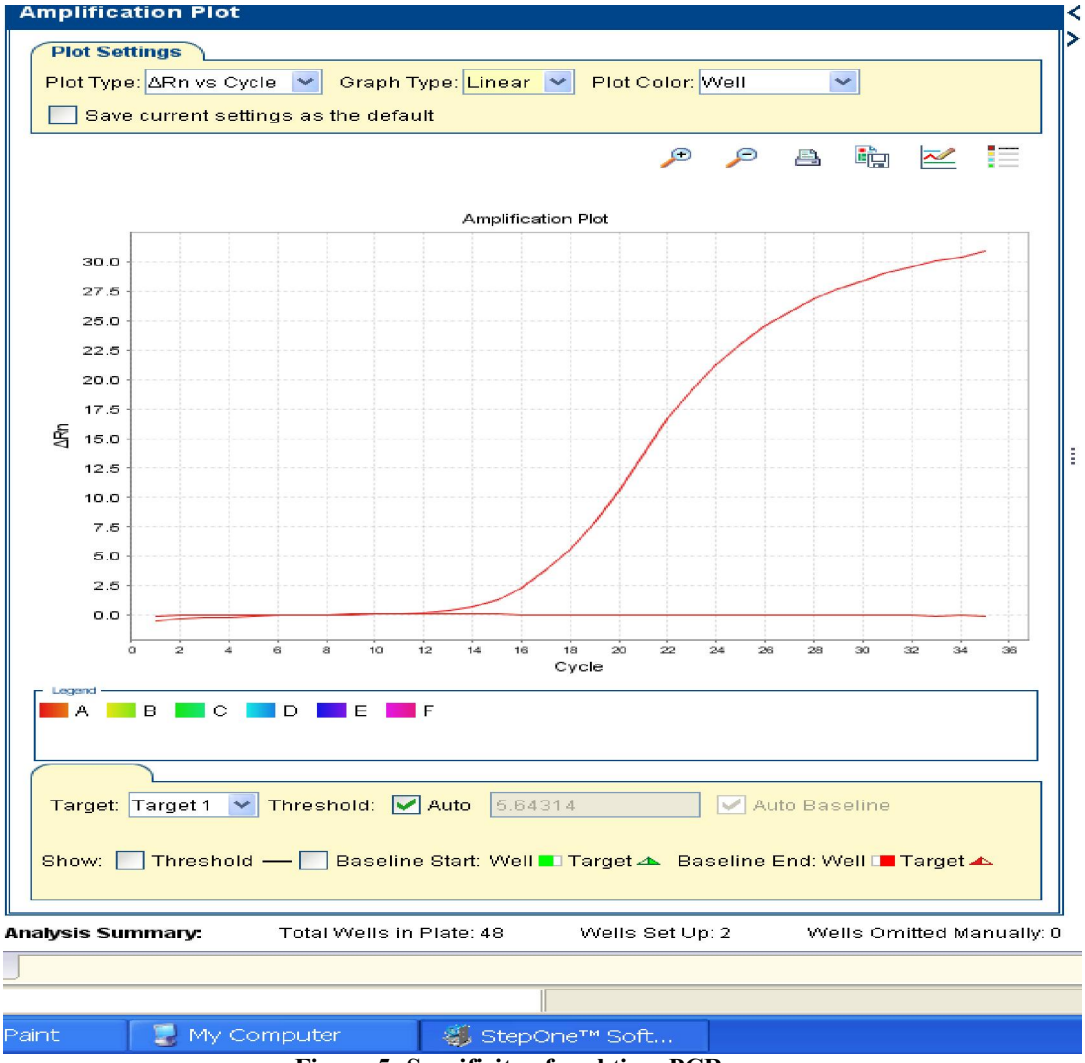


Figure 5: Specificity of real time PCR assay

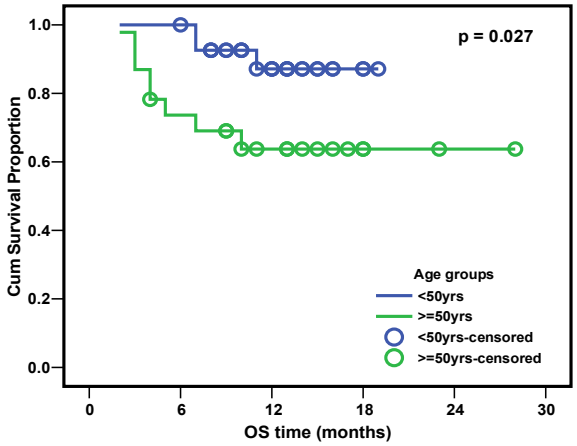


Figure (6). Overall survival and age in solid tumors

4.Discussion:

JC polyomavirus is widespread throughout the human population with 80% of adults exhibiting antibodies specific for JCV. Infection is thought to occur during early childhood and is usually subclinical. Under immunosuppressive conditions JCV can emerge from latency and cause severe diseases. Several reports have reviewed involvement of JC polyomaviruses in brain and non brain tumors [20]. Therefore, we found it is quiet relevant to establish sensitive diagnostic tool can determine and identify type of polyomavirus and measure viral load as quantitation of viral load will not only be helpful in giving potential information on viral burden and disease progression but also may influence the choice of initial antiviral therapy and tailor treatment regimens to reduce risk of viral mutation and subsequent viral resistance. However, in case of polyomaviruses infection, antiviral therapy is not available but infected patients were strictly followed up by monitoring viral load, this may assist in management of viral infection and save patient from severe complications. In addition, detection of viral load can be useful to classify infections as

asymptomatic or associated with clinical disease, and then can predict patients at higher risk of fatal outcome due to disease [21].

In this context, sensitive and specific snPCR tool has been established in our virology and immunology lab, NCI, Cairo University, according to the previously published protocol [18, 22]. Sensitivity and specificity of our PCR assay had been evaluated for detection and identification of polyomaviruses infection in patient's sera with a sensitivity level of 100 copies/µl for qualtitative snPCR and 10 copies/µl for quantitative PCR assays.

In the current study polyomaviruses DNA was detected in the sera of 28 patients, 33% (17/51) by qualitative first round PCR and JC polyomavirus DNA was detected in 11.8% (6/51) by snPCR assay. Moreover, polyomaviruses (PoV) DNA were detected in 23/51 (45%) by quantitative real time PCR assay. Out of 23 positive patients JCV was detected in 5 patients (22%). Our results were higher than that obtained by Marinelli and his/her coworkers [23] who reported presence of PoV DNA in 22.5% (28/120) among immunocompromised patients but were much lower than that obtained by Castini and his/her teamwork [24]. Their study was performed on colorectal cancer patients tissue registering 88.9% (16/18) occurrence of PoV infection. Such discrepancies may be attributed partially to difference in sample size and type of cancer disease and sensitivity of PCR assay. Moreover, concerning presence of JC polyomavirus infection, our results were lower than that reported previously who detected JCV in 81.4%, 77% and 48% respectively in their studies on colorectal cancer patients (CRC) [25, 26,27]. They concluded that JCV infection could be used as a non invasive hallmark for diagnosis of JC related CRC.

Regarding evaluation of our snPCR assay, our results revealed that, 7 cases who showed positive results by 1st round PCR were negative by semi nested assay (p=0.481), This could be attributed that those cases are suspected to be infected with other type of polyomaviruses rather than JCV. In such cases sequencing analysis is required to identify type of polyomaviruses among Egyptian cancer patients and to confirm sensitivity of the established diagnostic snPCR tool. But one case that was negative by quantitative RT-PCR could be detected by snPCR (p=0.027). Such results and when comparing both assays snPCR versus quantitative real time PCR assays on other samples (results are not shown), we suggested that in order to diagnose polyomavirus infection, first apply snPCR assay to identify and type polyomavirus and then quantitative PCR assay to measure viral load. Therefore, this will be applied soon as a routine at our diagnostic laboratory.

The viral load detected by quantitative assay showed relatively high level, ranged from 10 to $85x10^6$ with a median viral load of 200 copies/µl. We proposed our explanations as polyomaviruses rely on cellular enzymes and cofactors for DNA replication and these proteins are expressed in S phase. Since JCV spread by replication in lymphoid cells, such cells are not affected by malignancy and are functioning well in solid tumor patients. Therefore, polyomaviruses infection in solid tumor patients can lead to lytic infection with viral amplification [28].

Peripheral blood mononuclear cells are site of replication for JCV [26]. Therefore, further study is required to investigate presence of polyomaviruses and JCV in peripheral blood leukocytes to provide an accurate seroprevalence results.

Our results showed that infection of JC polyomavirus was not correlated with demographics or some clinical or biochemical parameters such as age, gender, stage of disease, tumor sites which was consistent with other studies [29,30]. It has been demonstrated association between immunosuppression status of cancer patients and an increased susceptibility to infection. This almost was observed in our results where solid tumor patients who were infected with polyomaviruses had leucopenia when compared to those without viral infection.

Regarding presence of JC polyomaviruses and status of disease, our results showed higher percentage of JC polyomaviruses were present among relapsed cancerous patients 6/14 (42.9%). This may be considered another evidence supporting association of polyomaviruses infection with immunosuppressive status of disease. Those patients showed to be at higher risk of severe polyomaviruses associated diseases and should be strictly monitored by measuring viral load during course of cancer treatment to manage viral infection.

Regarding outcome of disease, our results showed that 5/11 (45%) died patients were infected with JC polyomaviruses. In the current study, all studied cancer patients were subjected for one year overall survival analysis to investigate impact of some significant parameters on survival of our cancerous patients. Our results showed that, age had an impact on overall survival of solid tumor patients. This is matched with what is known about aging and its impact on efficiency of T cell effectors functions such as cytotoxicity or B-cell help in immunocompetent individuals. Decreased antibody production and a shortened immunological memory are the consequence [31]. In cancer patients, aging may cause further suppression of T-cells and PMNL [32], this lead to an increased susceptibility to infectious disease.

However, presence of polyomaviruses infection didn't show an impact on overall survival, but we

suggest that presence of polyomavirus infection could have an indirect effect on overall patient's survival via its suppressive effect on effector T cells compartment which in turn worsening immunological status of patients and then affect outcome of the disease.

Therefore, we suggest that the susceptibility of cancer patients to polyomaviral complications or other microbial superinfection could be affected directly by old age, cancer disease and indirectly by suppression of total leukocytes.

Disclosure

The authors report no conflicts of interest in this work.

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