

Tatiana Rasteiro Coelho

**Assessment of JC polyomavirus in normal colorectal mucosa,
hyperplastic polyps and sporadic adenomas and adenocarcinomas in a
*Portuguese population and its association with cancer development***

Dissertação de Doutoramento na área científica de Ciências da Saúde orientada pelo Senhor Prof. Doutor Luís
Manuel da Costa Marques de Almeida e apresentada à Faculdade de Medicina da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

**ASSESSMENT OF JC POLYOMAVIRUS IN NORMAL COLORECTAL
MUCOSA, HYPERPLASTIC POLYPS AND SPORADIC ADENOMAS AND
ADENOCARCINOMAS IN A PORTUGUESE POPULATION AND ITS
ASSOCIATION WITH CANCER DEVELOPMENT**

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"Dificuldades e obstáculos são fontes valiosas de saúde e força..."

Albert Einstein

"A verdadeira ciência ensina sobretudo a duvidar e a ser ignorante."

Miguel de Unamuno y Jugo

Ao meu filho e ao meu companheiro de vida...

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ABREVIATURAS E SÍMBOLOS

A – Adenine
AIDS – Acquired Immunodeficiency Syndrome
APC – Adenomatous Polyposis Coli
Arg – Arginine
ATL – Adult T-cell Leukaemia
AVRC - Arrhythmogenic Right Ventricular Cardiomyopathy
BKV – BK Virus
b.p. – Base Pairs
C – Cytosine
°C – Centigrade
CHC – Centro Hospitalar de Coimbra, EPE
CHD – Congenital Heart Disease
CI – Confidence Interval
CIN – Chromosomal Instability
CNS – Central Nervous System
CRC – Colorectal Cancer
DNA – Deoxyribonucleic acid
dNTPs – Deoxynucleotide Triphosphates
EBV – Epstein-Bar Virus
EDTA – Ethylenediaminetetraacetic Acid
Etc. – *et cetera*
Ex – Exon
G – Guanine
g – Gravitational Acceleration
Gly – Glycine
5-HT_{2A}R – 5HT Serotonin 2A Receptor
HBV – Hepatitis B Virus
HCG – Human Chorionic Gonadotropin
His – Histidine
HIV – Human Immunodeficiency Virus
HPV – Human Papillomavirus
HRR – Homologous Recombination Directed DNA Repair
HTLV-1 – Human T-lymphotropic Virus 1
HUC – Hospitais da Universidade de Coimbra, EPE

Int – Intron
Ins – Insertion
JCV – Human Neurotropic Polyomavirus JC or JC Virus
JCV+ – JCV positive
JCV- – JCV negative
KCl – Potassium Chloride
KH₂PO₄ – Dipotassium Hydrogen Phosphate
M – Molar
Mg²⁺ – Magnesium
ml – Millilitres
mm – Millimetres
mM – Millimolar
mRNA – Messenger Ribonucleic Acid
MS – Multiple Sclerosis
n – number
NaCl – Sodium Chloride
Na₂PO₄ – Disodium Phosphate
NES – Nuclear Export Signals
ng – Nanograms
NLS – Nuclear Localization Signals
OD – Odds Ratio
O/N – Over Night
ORI – Origin of Replication
P72 – Proline variant
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
pH - power of hydrogen
PML – Progressive Multifocal Leukoencephalopathy
pmol – Picomoles
rpm – Revolutions *per* minute
R – Adenine/Guanine
R72 – Arginine variant
RNA – Ribonucleic Acid
RT – Room Temperature
SCID – Severe Combined Immunodeficiency
SNP – Single Nucleotide Polymorphism
SV40 – Simian Virus 40

T – Thymine
TAE – Tris-acetate-EDTA
T-Ag or LT-Ag – Large T-antigen
t-Ag – Small t-antigen
TCR – Transcriptional Control Region
Tris-HCL – Tris-Hydrochloride
UK – United Kingdom
USA – United States of America
UTR – Untranslated Region
UV – Ultraviolet
v – Volume
VP1 – Viral Protein 1
VP2 – Viral Protein 2
VP3 – Viral Protein 3
vs. – *Versus*
WHO – World Health Organization
XMRV – Xenotropic Murine Leukaemia Virus-Related Virus
 μ l – Microliters
 μ g – Micrograms
 μ M – Micromoles
´ – Minutes
`` – Seconds
% – Percent

RESUMO/ABSTRACT

RESUMO

O poliomavírus neurotrópico humano JC (vírus JC) infeta de forma persistente e subclínica o trato gastrointestinal e urinário de cerca de 60-80% da população adulta mundial e é conhecido como o agente etiológico da Leucoencefalopatia Multifocal Progressiva em indivíduos imunocomprometidos. Recentemente, diversos estudos documentaram a deteção do genoma do vírus JC em amostras de tumores humanos quer de origem neural quer de origem não neural. De facto, sequências nucleotídicas do vírus JC e respetivas proteínas virais têm sido detetadas em diversas neoplasias humanas, incluindo no cancro colorretal. A oncogenicidade do vírus JC parece estar associada com a atividade da oncoproteína antigénio-T grande nas células hospedeiras. O antigénio-T do vírus JC tem a capacidade de desregular o controlo do ciclo celular, interagindo com proteínas supressoras de tumor, como a p53 e a pRb, o que o torna impar na capacidade de simultaneamente comprometer a integridade cromossómica e inativar os pontos de controlo do ciclo celular. Adicionalmente, o antigénio-T tende a autopropagar-se nas células infetadas e a imortalizar as transformadas, mediante interação com diversas outras proteínas celulares, tais como a IRS-1 e a beta-catenina.

Não estando ainda estabelecida uma relação causa-efeito direta, são necessários estudos adicionais, sistematizados e bem concebidos, com o intuito de esclarecer o papel do vírus JC no desenvolvimento de cancro colorretal, na medida em que poderá ser um alvo de estratégias preventivas e terapêuticas na oncogénese colorretal.

Atendendo ao facto de que o cancro colorretal apresenta uma elevada taxa de incidência na população Portuguesa, sendo a primeira causa de morte por neoplasia maligna, e que não foram, até à data, relatados estudos nesta matéria, o presente trabalho teve como principal objetivo esclarecer o papel que o vírus JC poderá desempenhar no desenvolvimento do cancro colorretal na população Portuguesa. Neste sentido, procedeu-se à avaliação: a) da frequência da infeção por vírus JC na mucosa colorretal, quer normal quer hiperplásica (pólipos), displásica (adenomas) e neoplásica (adenocarcinomas), e concomitante eliminação do vírus através da urina, numa população adulta Portuguesa padrão, b) da potencial associação entre a presença do vírus no cólon e o desenvolvimento, a gravidade e o grau das lesões colorretais, utilizando um painel de lesões com diferentes graus de hiperplasia/displasia/neoplasia, e c) do estado mutacional do gene TP53, de forma a rastrear potenciais alterações genéticas que possam atuar como co-fatores de risco na carcinogénese colorectal mediada pelo vírus JC em indivíduos infetados.

Foram estudadas amostras de mucosa colorretal e de urina de 100 doentes (grupo de estudo) e de 100 indivíduos controlo (grupo controlo) com indicação para realização de biópsia ou cirurgia colorretal para diagnóstico/tratamento de patologias colorretais. A seleção dos indivíduos a incluir no grupo de estudo e no grupo controlo foi efetuada durante os procedimentos colonoscópicos e mediante a presença ou ausência de lesões colorretais.

A deteção das sequências de DNA da estirpe Mad-1 do vírus JC foi efetuada por Nested-PCR, em DNA genómico obtido a partir das amostras de mucosa colorretal e de urina de ambos os grupos, utilizando dois conjuntos de *primers* complementares à sequência do gene viral que codifica a proteína antigénio-t pequeno. O rastreio mutacional do gene TP53 foi realizado por PCR seguida de Sequenciação Direta, utilizando DNA genómico obtido das amostras de mucosa colorrectal, quer normal quer displásica/neoplásica, de indivíduos infetados e não infetados pelo vírus JC.

A presente abordagem experimental permitiu a deteção de sequências de DNA do vírus JC (JCV+) em 169 dos 200 indivíduos estudados (84,5%). O DNA viral estava presente em 90 dos 100 doentes (90%) com lesões colorretais: em 23 de 26 (89%) pólipos hiperplásicos, 39 de 42 (93%) adenomas tubulares, 7 de 7 (100%) adenomas vilosos, 9 de 11 (82%) adenomas túbulo-vilosos e 12 de 14 (86%) adenocarcinomas. Neste grupo, sequências de DNA viral foram também detetadas em 48 das 100 (48%) amostras de mucosa normal adjacente, todas elas contíguas a lesões positivas para a presença do vírus, e em 41 de 100 (41%) amostras de mucosa normal não-adjacente. Em 60 das 100 (60%) amostras de mucosa normal dos indivíduos do grupo de controlo não foi detetado DNA viral (JCV-). Identificaram-se ainda sequências de DNA do vírus em 39 das 90 (43%) amostras de urina colhidas, sem diferenças estatisticamente significativas entre as estimativas de risco calculadas para a eliminação do vírus pela urina em ambos os grupos (48% para o grupo de estudo e 37% para o grupo controlo).

Num total de 81 amostras de mucosa colorretal normal infetadas pelo vírus JC, de 40 controlos e 41 doentes, a análise mutacional do gene TP53 permitiu a deteção de cinco alterações genéticas distintas (11818insC Int2, 11827G> C Int2, 11875insC Int2, 13399A> G EX6 e 19037insC Ex11, esta última ainda não descrita até à data) em 10 dos 40 controlos (25%) e 30 dos 41 doentes (73%). Nas 119 amostras de mucosa colorretal normal, negativas para a presença de DNA viral (60 controlos e 59 doentes), foi possível a deteção de uma única alteração no gene TP53 (11875insC Int2) em 5 dos 60 controlos (8%) e 13 dos 59 doentes (22%). Além disso, 74 amostras de mucosa colorretal displásica/neoplásica, 60 adenomas (55 JCV+ e 5 JCV-) e 14 adenocarcinomas (12 JCV+ e 2 JCV-), foram rastreadas para variantes do gene TP53 e quatro alterações genéticas concomitantes foram encontradas (11827G>C Int2, 12139C>G Ex4, 13399A>G Ex6 e a 19037insC Ex11) em todas as amostras estudadas.

Os resultados do presente estudo parecem refletir uma condição em que as células transformadas do epitélio colorretal oferecem um ambiente mais permissivo à replicação do vírus JC, sendo a infecção efetiva mesmo nos estadios mais precoces, como por exemplo nos pólipos hiperplásicos. O vírus JC parece apresentar um tropismo específico pelas células epiteliais com algum tipo de predisposição prévia e propensas a transformação oncogénica, parecendo haver seleção clonal das células infetadas.

Além disso, este estudo permitiu revelar a extensão epidemiológica da infecção pelo vírus JC, no trato gastrointestinal e urinário de uma população Portuguesa normal, e considerar a ocorrência de uma seleção biológica, das células infetadas pelo vírus, durante a tumorigénese em indivíduos com lesões colorretais.

Adicionalmente observou-se que as alterações genéticas mais relevantes encontradas no gene TP53, tais como o novo polimorfismo identificado na região 3'-UTR (19037insC Ex11) e a variante do codão 72 (12139C> G Ex4), são uma característica comum e exclusiva das células do epitélio colorretal de indivíduos infetados pelo vírus JC, especialmente aqueles com história clínica de displasia/neoplasia. Estas variantes do gene da p53 poderão, de alguma forma, estar relacionadas com a mudança do risco de desenvolvimento de cancro colorretal na presença do vírus JC. No entanto, a sua implicação no processo de carcinogénese colorretal mediada pelo vírus requer esclarecimentos adicionais.

ABSTRACT

The human neurotropic polyomavirus JC (JCV) infects, persistent- and sub-clinically, the gastrointestinal and urinary tract of 60-80% of the world adult population and causes Progressive Multifocal Leukoencephalopathy in immuno-compromised patients. Recently, a number of reports have documented the detection of JCV genome in samples derived from several types of neural and non-neural human tumours. In fact JCV DNA sequences and proteins have been detected in several human cancers, including in colorectal cancer (CRC), and its oncogenicity depends on viral large T-antigen oncoprotein (T-Ag) activity in human cells. JCV T-Ag can deregulate control of the cell cycle by interacting with the tumour suppressor proteins p53 and pRb, making it unique in the ability to simultaneously disrupt chromosomal integrity and inactivate cell cycle checkpoints. Additionally, it tends to self perpetuate within infected cells and to immortalize the transformed ones, cooperatively with a broad range of other cellular proteins, such as IRS-1 and beta-catenin.

Since there is not a direct cause-effect relationship, systematic and well-designed studies are necessary to clarify the role of JCV in colorectal cancer, particularly because it can be the target of preventive measures for this type of cancer.

Attending the fact that CRC has a high incidence rate in the Portuguese population, being the first leading cause of death by cancer, and that no data have been reported on this matter, this research work aimed to clarify whether JCV might have a potential role in the colorectal cancer development in the Portuguese population. Thus, we assessed: a) the frequency of the JCV infection in the colorectal mucosa, either normal or with hyperplastic (polyps), dysplastic (adenomas) or neoplastic (adenocarcinomas) colonic lesions, and the viral shedding through urine, in a standard adult Portuguese population; b) the potential association between the presence of the virus in the colon and the lesions development, severity and grade, using a panel of lesions with different grades of hyperplasia/dysplasia/neoplasia; and c) the mutational status of the TP53 gene in order to evaluate genetic alterations that might act as risk modifiers in JCV-mediated colorectal carcinogenesis in infected individuals.

We studied the colorectal mucosa and urine of 100 patients (study group) and 100 healthy individuals (control group) with indication to perform colorectal biopsy or surgery for diagnosis/treatment of colorectal disorders. The selection between the study group and control group was made during colonoscopic procedures, depending on the presence or absence of colorectal lesions.

In order to detect JCV Mad-1 strain DNA sequences Nested-PCR was performed in total genomic DNA obtained from both normal and abnormal colorectal mucosa and urine from both groups, using two primer sets targeted to the viral gene that encodes the small t-antigen.

The mutational screening of the TP53 gene was performed by PCR amplification, followed by DNA Sequencing, using total genomic DNA obtained from either normal or dysplastic/neoplastic colorectal mucosa samples from JCV infected and non-infected individuals.

We were able to detect viral nucleotide sequences in 169 of the 200 studied individuals (84.5%). JCV DNA sequences were present (JCV+) in 90 of the 100 patients (90%) with colorectal lesions: in 23 of 26 (89%) hyperplastic polyps, 39 of 42 (93%) tubular adenomas, 7 of 7 (100%) villous adenomas, 9 of 11 (82%) tubulovillous adenomas and 12 of 14 (86%) adenocarcinomas. JCV DNA was also detected in 48 of the 100 (48%) normal adjacent mucosa samples, all of them adjacent to positive colorectal lesions, and in 41 of the 100 (41%) normal non-adjacent mucosa samples. No viral DNA (JCV-) was detected in 60 of the 100 (60%) normal mucosa samples of the control group. We also detected JCV DNA sequences in 39 of the 90 (43%) collected urine samples, with no statistically significant differences between estimates of risk of viral shedding in the urine of both groups (48% for the study group and 37% for the control group).

In a total of 81 normal colorectal mucosa specimens infected by JC virus, from 40 controls and 41 patients, the mutational analysis of the TP53 gene allowed the detection of five different genetic alterations (11818insC Int2, 11827G>C Int2, 11875insC Int2, 13399A>G Ex6 and a new 19037insC Ex11) in 10 of the 40 controls (25%) and 30 of the 41 patients (73%). Furthermore, from the 119 normal colorectal mucosa samples with no positivity for JCV DNA (60 controls and 59 patients) we were able to identify a single TP53 defect (11875insC Int2) in 5 of the 60 controls (8%) and 13 of the 59 patients (22%). Furthermore, 74 dysplastic/neoplastic colorectal lesions were screened for TP53 variants, 60 adenomas (55 JCV+ and 5 JCV-) and 14 adenocarcinomas (12 JCV+ and 2JCV-) from patients included in the study group, and four concomitant TP53 genetic alterations were found (11827G>C Int2, 12139C>G Ex4, 13399A>G Ex6 and 19037insC Ex11) in all the studied samples.

Our observations may reflect a condition where the transformed colorectal epithelial cells offer a more permissive environment for JC virus replication, being effective even since the hyperplastic polyps not prone to carcinogenesis. JCV may have a specific tropism for epithelial cells with some inherent predisposition and prone to oncogenic transformation, with selection of the infected ones.

Furthermore, we propose the epidemiological extent of the JCV infection in the gastrointestinal and urinary tracts of a normal Portuguese population, and the occurrence of a biological selection of JCV infected cells during tumourigenesis in individuals with colorectal lesions.

Additionally, we propose that the most relevant genetic alterations found in TP53 gene, such as the new single nucleotide polymorphism identified in TP53 3'-untranslated region (19037insC Ex11) and the codon 72 variant (12139C>G Ex4), are a common and exclusive characteristic of the colorectal epithelium cells of JCV infected individuals, especially those with clinical history of dysplastic/neoplastic colorectal lesions. These p53 variants can be associated, in some extent, with the risk modification of JCV-mediated colorectal cancer development. However, its implication in the JCV-mediated colorectal carcinogenesis requires further elucidation.

CHAPTER 1

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HYPOTHESIS

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JC virus in the pathogenesis of colorectal cancer, an etiological agent or another component in a multistep process?

Tatiana R Coelho¹, Luis Almeida¹, Pedro A Lazo^{2*}

Abstract

JCV infection occurs early in childhood and last throughout life. JCV has been associated to colorectal cancer and might contribute to the cancer phenotype by several mechanisms. Among JCV proteins, particularly two of them, large T-antigen and agnoprotein, can interfere with cell cycle control and genomic instability mechanisms, but other viral proteins might also contribute to the process. Part of viral DNA sequences are detected in carcinoma lesions, but less frequently in adenomas, and not in the normal surrounding tissue, suggesting they are integrated in the host cell genome and these integrations have been selected; in addition viral integration can cause a gene, or chromosomal damage. The inflammatory infiltration caused by a local chronic viral infection in the intestine can contribute to the selection and expansion of a tumor prone cell in a cytokine rich microenvironment. JCV may not be the cause of colorectal cancer, but it can be a relevant risk factor and able to facilitate progression at one or several stages in tumor progression. JCV transient effects might lead to selective expansion of tumor cells. Since there is not a direct cause and effect relationship, JCV infection may be an alternative to low frequency cancer predisposition genes.

Cancer is a multifactor disease that its progression is determined by several genetic alterations, which are most likely sequentially selected for their contribution to the tumor phenotype [1]. This phenotypic complexity makes difficult to determine the specific roles for biological agents that might be considered carcinogenic, and even more difficult to determine their causality, or implication at a particular stage in disease progression. Among the exogenous agents associated to cancer initiation or progression are chemicals, which are single molecules with a specific effect; and some infectious agents, including viruses and bacteria.

Infectious diseases are acquiring relevance as important pathogenic elements in human cancer, since almost one fifth of human cancers are associated with infectious agent, either bacteria or viruses, particularly in the gastrointestinal tract [2], but do not represent mainstream oncologic research. The development of several types of human cancers can be triggered by the

exposure to different infectious agents, viruses or bacteria, such as Human Papilloma Virus (HPV) infection associated to cervical carcinoma [3], hepatitis B virus to liver carcinoma [4], Epstein-Barr virus to Burkitt lymphomas [5], HTVL-1 to ATL [6], *Helicobacter pylori* to gastric carcinoma [7] and more recently a human retrovirus, XMRV, has been associated to sporadic prostate cancer [8-10]. More recently, it has been reported the association between the development of lower gastrointestinal tract neoplasias and infectious agents [7], such as between colorectal cancer (CRC) and JCV infection. JCV is a virus very well adapted to humans, thus its widespread infection and adaptation to humans complicates the determination of its etiologic contribution to cancer development, and it has also been associated to some neurodegenerative diseases [11].

A chronic infection might be a non genetic alternative to cancer predisposing genes. The mechanistic role of infectious agents is even more complex, because they might contribute in several different ways to oncogenesis. Infections can also play a role at different stages in tumor progression, from initiators to promoters of the process, depending on the phenotypic aspect to which

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they can contribute and some might be transient or a consequence of the host response to chronic infection. Thus, causality of infectious agents in cancer is somewhat more difficult to demonstrate.

The viral contribution to oncogenesis has to be addressed by determining how the viral life cycle and the host response, including their alterations, can be integrated within the multistep process of tumor development. Viruses that are well adapted to humans can participate in oncogenesis process because they have not undergone a negative evolutionary pressure, since cancer develops at an age well above the median age of humans till the twentieth century, However, the benefits of interfering or preventing infectious agents implicated in cancer is very large as demonstrated in hepatic, gastric and cervical carcinomas [2].

Colorectal cancer (CRC) is the third most common tumor in women and the fourth in man; representing annually one million new cases worldwide, and more than 500.000 deaths are caused by this malignant disease [12]. Most colorectal cancers are sporadic in their origin and associated to different risk factors, such as a diet rich in fat and animal protein intake [13].

JCV infection in human population

The extent of the exposure to JCV infection is indirectly known by seroprevalence rates detected in different populations throughout the world, ranging from 44 to 90% in the USA, UK, Germany, Brazil and Japan [7,14,15]; all indicating it is widespread and common. The infection may occur by fecal contamination and is usually persistent and sub clinical, but is reactivated under conditions of immunosuppression, such as in patients with AIDS, and JCV can also emerge from latency and become a lytic infection causing progressive multifocal leukoencephalopathy (PML) [16]. JCV infection occurs relatively early during childhood and seropositivity increases with age, and may be as high as 50% by the age of 10 [17]. Infection appears to occur through the gastrointestinal tract [18-20], but also it has been detected in the respiratory tract raising the possibility of an oral-fecal transmission [21,22]. The detection of antibodies from childhood to adults suggests that these antibodies are unlikely to be protective, however they are a clear indicator of exposure, probably multiple or sustained, to JCV. The persistence of seropositivity does not mean that individual patients have an active infection at the moment, or that harbor viral DNA or latent virus in some cells. The antibody mediated immune response is unlikely to be protective, and control of JCV infection is more probably performed by T-cell mediated responses, if successful [23]. It would be interesting to determine if tumor-infiltrating lymphocytes

from colon carcinoma can recognize and react with JCV infected cells.

Alternatively, urinary secretion of JCV has been used as an alternative detection method. JCV was present in 24% of the samples out of 498 healthy individuals, and the proportion increased with age [24]. However, in immunocompromised patients there is a significant increase in JCV viruria, although it does not correspond with the level of immunosuppression, suggesting the presence of a subclinical infection that might require additional factors to be reactivated [25]. These data indicate that most people have been exposed for some time to this virus in order to generate an immune reaction. In cancer patients viruria was detected in up to 70 percent of the cases, but viral DNA could not be detected in carcinoma tissue; but this apparent discrepancy might have a methodological explanation, as primer selection may not be suitable in case of partial loss of viral DNA, or viruses might transiently contribute to a particular stage of tumor progression [14]; particularly since later several studies were able to detect viral DNA in carcinoma samples [15,26].

The JC virus

The polyomaviruses family comprises SV40, JCV and BKV viruses, the primate SV40 virus is the reference member, while BKV and JCV are much poorly characterized, and mostly done with SV40 specific cross-reactive reagents [27]. Despite their isolation from humans, their role in human pathology is far from clear for both JCV and BKV. JCV is a nonenveloped virus with double-stranded DNA that forms minichromosomes with cellular histones. The JCV early region encodes two oncoproteins, large T antigen (T-Ag) and small t (t-Ag) antigen and the late region encodes three capsid proteins (VP1, VP2 and VP3) and a small regulatory protein (Agnoprotein). The viral DNA is packed as a non-enveloped virus in a virion composed of 72 pentamers of the major VP1 capsid protein, that also includes one unit of the minor VP2 and VP3 proteins [11]. These early viral proteins have a high transformation and oncogenic potential in experimental systems [28,29]. There are two possible outcomes to JCV infection: permissive cells, as oligodendrocytes, are able to support viral DNA replication resulting in a lytic infection; in nonpermissive cells, as those of the colorectal epithelium, resulting in silent or abortive infection, or probably in cell transformation and cancer [28]. Despite these evidences, the role of JCV in human malignancies, and of its oncoproteins in promoting transformation of cells in vitro and in vivo, is still far from clear. JCV does not infect experimental animals, and its roles have been implied by analogy to SV40 [30].

JCV is a human neurotropic polyomavirus, and neurological diseases, such as progressive multifocal leukoencephalopathy have been associated to JC virus [11,31]. Initial studies suggested that the transforming ability of JCV was limited to specific neural cell types, and that this property mapped to the noncoding regulatory sequence in the origin of DNA replication, but no neural tumor has been associated to JCV. However, JCV can also infect and transform different cell-types in culture and is highly oncogenic in several laboratory animal models [32]. Furthermore, there is evidence for the presence of the JCV genome in a broad range of human cell types and tissues. For example, the JCV genome has been detected in tonsillar stromal cells, B lymphoid cells, kidney epithelial cells, and upper and lower parts of the gastrointestinal tract, including the mucosa of the colon, which is considered as the natural human reservoir [18].

In addition, the JCV genome has variant forms which might behave differently from an oncogenic point of view. The Mad-1 strain, which lacks 98 nucleotides repeats in its transcriptional regulatory region, was the only one detected in colon carcinomas from California patients [33], but no functional study on the characteristics of this regulatory region has been performed. More recently in another study performed in Taiwan, most CRC cases appeared to have the JCV-CY genotype [15], which is also the dominant genotype in this population, although the Mad-1 strain was also detected [15]. Thus it is not clear if this strain association reflects the viral types circulating in different geographical areas, or if there are particular strains more strongly associated to carcinomas.

JCV DNA in normal, benign and malignant colorectal lesions

JCV DNA sequences and proteins have been detected in a broad range of human tumors of glial and non-glial origin, including gliomas, ependymomas and medulloblastomas, as well as in several non-neural clinical specimens of upper and lower gastrointestinal tumors, such as colorectal cancer (CRC) [7], suggesting they can infect a wide range of cell types, but the role of JCV in human malignancies is still unclear. A very important issue is to determine whether the presence of JCV presents any difference between tumor of different grades and its normal surrounding mucosa. Very few studies have been performed in this context, but a picture of the situation is emerging from three independent studies in Japan [26], Taiwan [15] and South Korea [34]. Remnant JCV large- T DNA was detected in 28-80% of the cases, but protein was only detected in 16% of the carcinomas. Also the frequency is lower in benign adenomas, and negative in normal surrounding tissue [26]. DNA corresponding to agnoprotein and VP genes was not detected suggesting they were deleted [34]. More

importantly in this study no viral DNA sequences were detected in the adjacent normal tissues [34]. The general findings are consistent among these three studies, and the variation in frequency is likely to be a consequence of the different primers used for viral DNA detection. However, a similar and earlier study in the USA was not consistent with this observation, but in that case there was no microdissection of the CRC biopsies and primers were different [14]. In none of these studies there is any indication about the JCV strain implicated.

The variability in JCV detection suggests that in an infected colon, in some cells there might be integration with partial loss of JCV DNA, which may have a pathogenic role in cancer development, probably permitting additional events that will lead to cancer progression by permitting selection of a cell subpopulation. When human CRC samples were grown as xenographs in nude mice that permit expansion of the cancer cell population, all of them resulted positive for JCV [18], suggesting that the cell subpopulation containing JCV might be selected for its growth and adaptation characteristics. In a way, this situation is reminiscent of what occurs in cervical carcinoma, in which most HPV infections and lesions regress, but some progress, and cervical carcinomas have remnant viral DNA coding for some viral proteins, E6 and E7, but the other HPV proteins are not expressed in these carcinomas [3]; these viral proteins participate in the oncogenic process, but clearly need additional, non virally related events [35,36].

Multiple effects of JCV large T- antigen in host cells

JCV is able to translate its early proteins, namely LT-Ag, in order to trigger the progression of the cell cycle to the phase S in host-cell [29,37]. JCV LT-Ag has the ability to interact with p53; thus it is possible that in some cells this interference with p53 [38], which also interferes with viral replication [38], might allow the occurrence of additional genetic damage representing a step forward in colon carcinogenesis [39,40]. In addition, there are several additional mechanisms by which LT-Ag can also interfere with cellular functions. JCV LT-Ag can interact with proteins involved in cellular regulation such as IRS-1 (Insulin receptor substrate-1), a major protein of the insulin-like growth factor I receptor (IGF-IR) signaling pathway, which is activated and translocated to the nucleus in the presence of LT-Ag [41]. Activated IRS1, is an adaptor in the cell response to insulin, activating PI3K, implicated in cell survival [42], and proliferation signals [43]. Thus at the same time it will permit survival and expansion of a JCV containing subpopulation. Some polymorphisms of the IRS1 gene have been associated with an increased risk of colorectal cancer [44]. These signaling effects associated to LT-Ag

can participate at any given stage of cancer progression, facilitating the expansion of a specific subpopulation, perhaps already pretumoral. LT-Ag can also inhibit homologous recombination directed DNA repair (HRR) causing DNA damage, mechanistically by its interaction with IRS1 [45], which also interacts with Rad51 at locations of damaged DNA [46,47], and thus may contribute to generate some genetic instability in cells containing JCV [48]. JCV also by a hit and run mechanism, that is a transient effect, is able to trigger genetic instability by interacting with p53 and β -catenin in colonic cells, which is detected only in the first seven days after infection [49]. LT-Ag also contributes to the stabilization of β -catenin by a novel mechanism mediated by the small GTPase Rac1 [50]. β -catenin is an integral component of the Wnt signaling pathway whose stabilization is associated with increased transcription of genes that regulate cellular proliferation, e.g., *c-myc* and cyclin D1, despite the fact that the functional consequence of this JCV interaction in cancer development remains to be elucidated. LT-Ag interacts with β -catenin [51], and β -catenin implication in colorectal cancer is well known [52,53]. This observation is supported by reports on the involvement of Wnt signaling pathway [54] and *c-myc* [55] in colorectal carcinogenesis. If the effect persists for some time in infected cells harboring JCV, they might contribute to expand a cell subpopulation that later might give rise to cancer.

Effects of JCV agnoprotein in host cells

JCV expresses a small 71 aminoacids protein, known as agnoprotein, which is a regulatory protein that can repress the expression of p21WAF-1/Cip1, a regulator of the cell cycle that inhibits the activation of cyclin/CDK complexes and releases E2F transcription factor from phospho-Rb, thus agnoprotein removes an inhibitor of cell cycle progression. Consequently, p21WAF-1/Cip1 and E2F stimulate several proteins, the function of which is essential for cell cycle progression and rapid cell proliferation [56]. This role is in some aspects reminiscent of the action of E6 in cervical carcinogenesis, where the elimination of p53 also results in lack of induction of p21 [57,58], and permits cell cycle progression. Agnoprotein can also alter the expression of Ku70 and Ku80 [56], two proteins implicated in DNA repair [59,60], and thus indirectly contribute to a potential accumulation of genetic damage. Furthermore, agnoprotein can also interact with the YB-1 transcription factor [61], a factor that when is downregulated results in an induction of apoptosis [62] by regulating the mTOR/Akt pathway [63]. This YB-1 transcription factor can also modulate the response to the erbB2 receptor [64], and contribute prevention of premature senescence [65]. In addition YB-1 also controls some chemokine ligand [66]

and metalloprotease gene expression, such as MMP13 [67], two types of proteins that can have an important role in carcinogenesis. YB-1 has been shown to have a predictive value in breast cancer patients identifying those with a poorer prognosis [68,69]. However, there is no systematic study of the presence and role of this viral protein, or of YB-1, at different stages of CRC progression. Agnoprotein has been shown to inhibit differentiation of oligodendrocytes [70]; if a similar effect could be induced in colonic epithelium, it will be an additional contributing factor towards tumorigenesis. But a detailed study of agnoprotein presence in biopsies representing different stages of CCR has not yet been performed.

Tumor immune microenvironment

Another important component of tumorigenesis is represented by the tumor microenvironment, where local cytokines that can play a stimulatory role. These cytokines may originate either in the tumor itself or in the local inflammatory infiltrate, and can activate JCV gene expression by a cis-acting transcription factor, *Egr1*; a factor which mostly activates the late promoter, and affects VP1 expression and viral replication [71]. The expression of this capsid protein can also trigger an inflammatory and immune reaction, with the corresponding local availability of several additional cytokines. This viral reactivation might also result in viral production, dissemination, and reinfection of neighboring cells. This mechanism could be important for maintenance or reactivation of a local subclinical infection. In this context it is important the recent observation that patients which have undergone liver transplantation have an increased risk of colorectal cancer due to reactivation of JCV, probably as a result of immunosuppressive treatment [72].

Additional aspects in JCV associated oncogenesis

Another mechanism by which JCV can contribute to colorectal carcinogenesis might, in some aspects, be similar to the role of human papillomaviruses (HPV) in cervical carcinoma [35,36]. Viral DNA integration has only been partially addressed in cervical cancer associated to HPV [73], and the chromosomal location of viral integration sites coincides with those of fragile chromosome sites [74], and with translocation breakpoints already detected in other types of carcinomas [36,75], although the cellular genes affected are not known. If the JCV infection is persistent, then there is a good probability that JCV DNA might integrate in the host cell genome, and some of the viral DNA remains in a manner similar to what occurs with HPV in cervical carcinoma [74]. JCV DNA integration appears to be a common observation in CRC when this issue has been

studied [26]; however its significance has not yet been properly addressed. This integration, if demonstrated, can explain why in some cases JCV is not detected. Integrated JCV DNA may be an important and irreversible component in the pathogenesis of CRC. In that way a cell population can harbor viral DNA from which some viral genes can be expressed with an altered regulation. The penetrance of the remaining viral gene and its level of expression might condition the risk of developing cancer. Also other viral genes might be lost, particularly those of the capsid, thus an immune reaction against them will not be effective.

Roles that JCV can play in colorectal cancer

The role of JCV in cancer does not fit within a classical concept of direct relation between cause and effect as it is for other infectious diseases and neither as a simple risk factor as applied to chemical or physical carcinogens. JCV might participate in different ways in the pathogenesis of colorectal cancer; both direct and indirect (Figure 1). This situation is a consequence of the complexity of the mechanisms contributing to cancer phenotype, which have many different phases, ranging from initiation, promotion, morphological progression with different biological characteristics, to tumor maintenance and dissemination. Thus, to pinpoint a unique mechanism of action for a virus represents a very simplistic approach to the problem. Some of the effects

induced by JCV might be transitory and contribute to tumor progression at a particular stage of tumor progression. For example, a transitory genetic instability will permit generation of generic damage that might facilitate progression, but once it occurs, it is no longer needed. Other contributing factors are viral protein expression or viral DNA integration that might be important only at some steps in cancer progression. Also chronic infection is known to be a bad prognostic indicator due to the immune cell infiltration and generation of a microenvironment very rich in cytokines, which can promote expansion of premalignant or malignant cells, not necessarily with JCV, that will facilitate cancer cell growth and dissemination. Some of the effects might be transient and this will further complicate establishing a direct relation between JCV and CRC. In any case there is no evidence, epidemiological or physiopathological, to rule out a role for JCV in colorectal carcinogenesis, even if its role is still undefined; but on the contrary there is enough information pointing to roles that remain to be conclusively established.

Outstanding issues and future prospects

The role of any virus as a causal agent in cancer should not be considered simply as a direct cause-effect. The virus should be considered as a complex agent with potentially multiple and varying effects. In order to properly establish if JCV is indeed an important risk

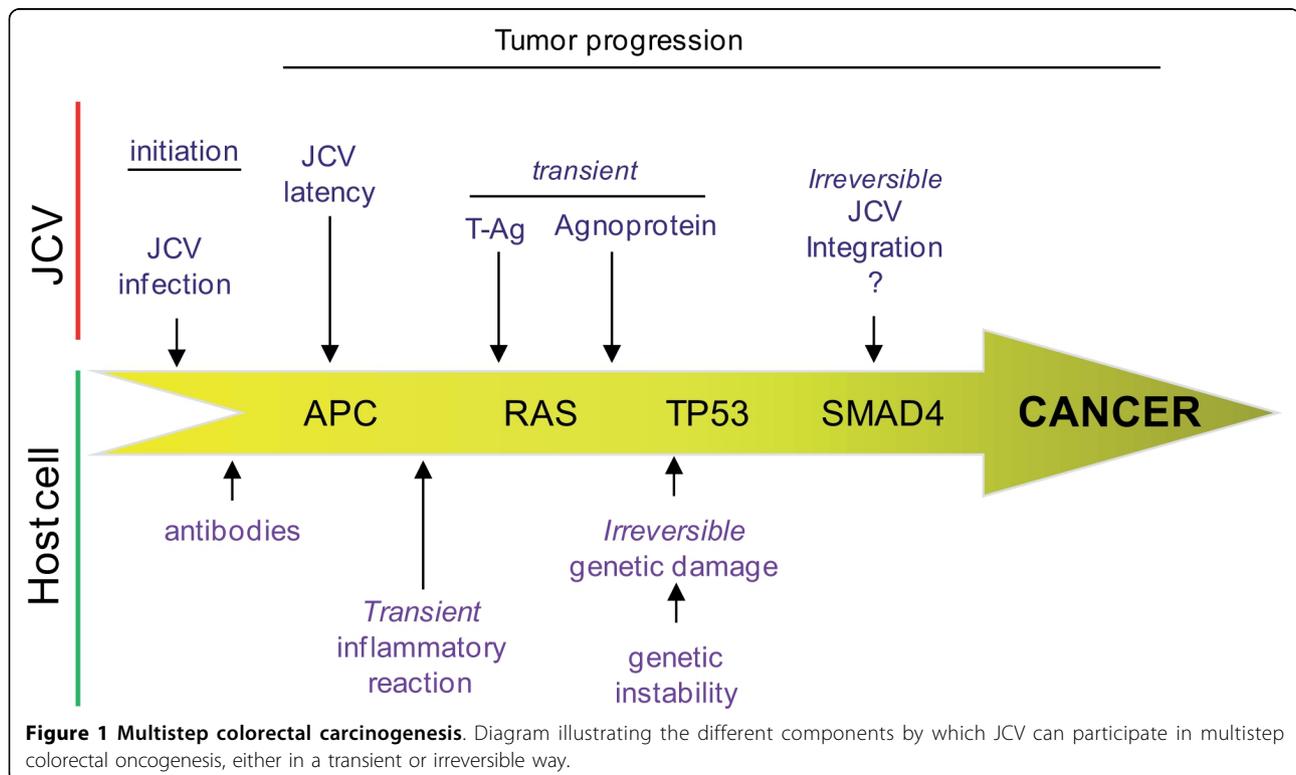


Figure 1 Multistep colorectal carcinogenesis. Diagram illustrating the different components by which JCV can participate in multistep colorectal oncogenesis, either in a transient or irreversible way.

factor for colorectal cancer there are several issues that need to be properly addressed.

1. Establish conclusively that CRC patients, in a significant number of cases, have been exposed to this virus, and of which an indirect marker will be the presence of specific antibodies.

2. Improve detection of viral DNA by using standardized sets of primers for each of the relevant viral genes, as well as strain identification. This should also contribute to establish the potential and irreversible integration of remnant JCV DNA in cancer cells, and permit identification of significant differences among malignant, benign or normal surrounding tissue.

3. Correlate JCV DNA presence with other mutations known to sequentially occur in CRC, and its association with other known risk factors.

4. Identification of viral protein expression at different stages of colorectal cancer progression. For this aim development of better specific antibodies for JCV proteins are necessary.

5. Characterize the host immune response to JCV in order to manipulate it and develop strategies to eradicate the virus from the human population. In particular determine the role T-cell responses, since the natural antibody response does not seem to be protective.

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Authors' contributions

TRC, LA and PAL conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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CHAPTER 2

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CHAPTER 2

2. Assesment of human JC polyomavirus presence in normal colorectal mucosa, hyperplastic polyps, sporadic adenomas and adenocarcinomas

2.1. Introduction

Infectious diseases are acquiring relevance as important pathogenic elements in tumour development, considering that almost one fifth of the human cancers are associated with the presence of infectious agents.

The underlying mechanisms relating the presence of microorganisms and cancer development are poorly understood in humans, but it is generally accepted that several types of cancers can be triggered by the exposure to different infectious agents, either viruses or bacteria[1,2]. The most relevant reports associate the Human Papillomavirus (HPV) with cervical carcinoma[3], Hepatitis B Virus (HBV) with liver carcinoma[4], Epstein-Barr Virus (EBV) with Burkitt lymphoma[5], Human T-lymphotropic virus 1 (HTLV-1) with adult T-cell leukaemia[6] and *Helicobacter pylori* with gastric carcinoma[7]. However, the role of polyomaviruses, such as Simian Virus 40 (SV40), BK virus (BKV) and the human neurotropic polyomavirus JC (JCV), in human cancers has been much more controversial.

JCV belongs to the *Polyomaviridae* family and it is the etiologic agent of the Progressive Multifocal Leukoencephalopathy (PML). The name polyoma is derived from the Greek terms: *poly-*, meaning “many”, and *-oma*, meaning “tumours”, and it refers to the ability of these viruses to cause different tumours. JC virus was first isolated in 1971 from the brain of a patient with PML. This virus, named Mad-1, is the JCV prototype strain and it has been used in most *in vitro* and *in vivo* studies of JCV (Diagram 2.1)[8].

JCV seems to infect, with rare exceptions, the gastrointestinal and urinary tracts of all human populations investigated till date. Most individuals become JC virus-infected during childhood or adolescence and, by adulthood, approximately 60–80% is seropositive. Although the exact mechanism of JCV transmission is unknown, it is thought to be spread via a fecal–oral route, since the virus can be detected in untreated urban sewages, and its presence is prevailing in the gastrointestinal and urinary tracts. Initial JCV infection is thought to occur in the tonsils, with posterior spreading to infect the epithelium of the kidney, where it establishes a life-long persistent and latent infection (Diagram 2.2)[9].

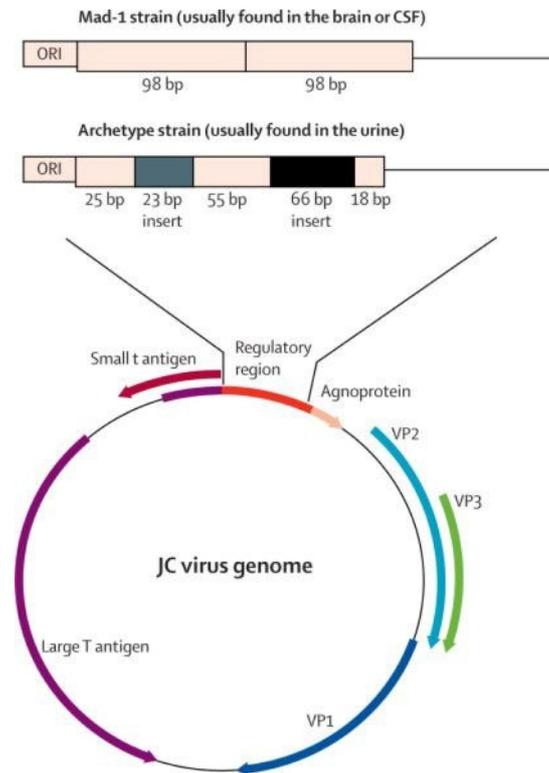


Diagram 2.1 - The JC virus (JCV) genome. The JCV genome is small (5.13 kb) and contains a limited coding capacity. There is a non-translated regulatory region known as the transcriptional control region (TCR) of about 400 b.p. that contains the origin of replication (ori) and the promoters and enhancers that control replication. The early genes (counterclockwise) encode two replication proteins, large T-antigen and small t-antigen, which are expressed soon after the virus enters the cell. The late region (clockwise) encodes the capsid proteins (VP1, VP2, and VP3) and a maturation protein (agnoprotein), and is expressed only after viral DNA replication begins.

The TCR of the Mad-1 strain of JCV contains two 98 b.p. tandem repeats which contain binding sites for various transcription factors. Large T-antigen is not a transcription factor *per se* but it can auto-regulate the early promoter as the replication cycle proceeds. When large T-antigen reaches a sufficient concentration in the cell, it binds to viral DNA which may block the assembly of functional transcriptional complexes, repressing early transcription. Large T-antigen indirectly contributes to activation of JCV late transcription, perhaps by stabilizing interactions among transcription factors.

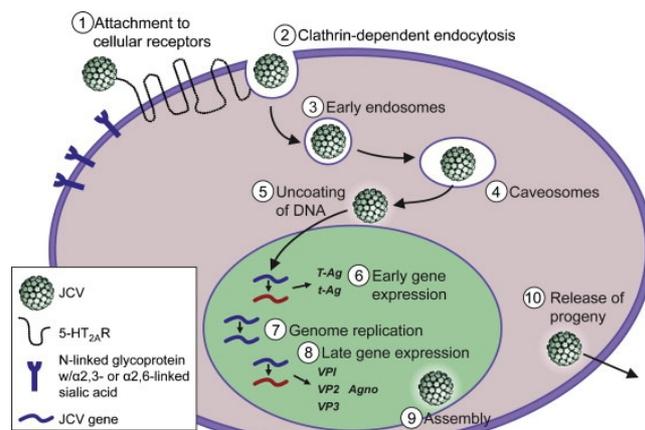


Diagram 2.2 - The JCV life cycle: JCV infection of host cells is initiated by attachment to cellular receptors, α -2,3- or α -2,6-linked sialic acid and serotonin receptor 5-HT_{2A}R; the virus is internalized into cells by clathrin-dependent endocytosis; JCV then traffics through early endosomes and caveosomes to the nucleus; in the nucleus viral early gene transcription occurs, followed by viral DNA replication and late gene transcription; after production of the viral structural proteins VP1, VP2, and VP3, progeny virions are assembled in the nucleus and released.

JCV infection in immuno-competent hosts is usually subclinical and apparently confined to the kidney. Still, it remains not clear whether JCV reactivation causes viral spread to the central nervous system (CNS) or if a latent infection in the CNS becomes locally reactivated. However, B-lymphocytes of the bone marrow and peripheral blood are permissive to JCV infection, suggesting that viral spread after primary replication may occur via a hematogenous route. In the CNS, JCV infects glial cells, including astrocytes and the myelin-producing cells, known as oligodendrocytes[10].

In immuno-compromised individuals JCV can become reactivated, leading to an enhanced viral replication and to lytic infection of the CNS, causing the destruction of oligodendroglia and resulting in the fatal demyelinating disease PML[11,12]. PML has been reported in individuals with immuno-suppression caused by human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS)[13], organ transplantation[14] or inherited immuno-deficiencies, such as severe combined immunodeficiency (SCID), hyper-immunoglobulinemia M and CD40 ligand deficiency. Recently, PML has been reported in patients with Multiple Sclerosis (MS) and Crohn's disease that are receiving the drug natalizumab, which blocks leukocyte transport from the gut to the brain[15].

Although JC virus does not infect hosts other than humans, recent reports state that JCV inoculation is able to induce neuronal tumours in experimental animal models. JCV can induce tumour formation in small rodents including hamsters[16]and rats[17], and in non-human primates, including owl monkeys[18] and squirrel monkeys[19]. The tumour type, origin and characteristics differ between host species and also depend on the route of inoculation and virus strain. Although JCV does not induce tumours in normal mice, the expression of the viral early region in transgenic mouse models can cause tumourigenesis[20].

The unequivocal oncogenic activity of one of JCV early proteins, the large T-antigen (T-Ag), has raised the possibility that JCV can also induce cancer development in human hosts[21]. JCV genome (Diagram 2.1) has been detected in several human pre-malignant lesions and in tumours, either of neuronal origin, such as medulloblastomas[22], oligoastrocytomas[23] and glioblastomas[24], or of non-neuronal origin, particularly in the different stages of colonic adenomatous lesions and in colorectal cancer (CRC)[25,26]. In colorectal neoplasias, it has been proposed that, under virus activation, the viral regulatory protein coded by T-Ag oncogene is, probably, the main key in the carcinogenic mechanism, since it was shown that it is responsible, in a carcinogenic multistep pathway, for chromosomal instability (CIN) by disrupting several host-cell signalling and regulatory processes[27].

2.2. Purpose

Even considering the bibliographic lack of consensus, the association between JCV infection of the colorectal epithelium cells and the development of CRC is still being suggested, but this hypothesis asks for further elucidation, especially at the populational level.

Colorectal cancer is the third most common cancer in the world. There is an estimative of 1.24 million of new cases worldwide, *per year*, and 500.000 deaths caused by this malignant disease. It comprises 10% of the global cancer burden and it is the most frequent in Australia, New Zealand, Western, Southern and Northern Europe and in North America, being considered as a disease of the Western lifestyle[28]

The wide geographical variation in CRC incidence across the world can be attributed to differences in diet, particularly the consumption of red and processed meat, fiber and alcohol, as well as bodyweight and physical activity. Incidence rates of CRC are increasing in countries where rates were previously low, especially in Japan and other Asian countries, as diets become more Westernized, and either gradually stabilizing in Northern and Western Europe or declining in North America[28].

Colorectal cancer incidence worldwide is noticeably higher in men than in women (rate ratio 1.4:1.0). In both sexes there are ten-fold differences in incidence between the different regions of the world[28].

In Portugal, CRC is highly incident, with over six thousand new cases *per year*, with a mortality rate approaching 50% and being the second leading cause of death by cancer, in both sexes[29]. Despite these high incidence and mortality rates no studies have been reported, so far, stating the JCV oncogenicity or even its occurrence among the Portuguese population. Considering the lack of reports on this matter, with this experimental approach we intended to assess: a) the prevalence rates of JCV infection in the colorectal mucosa, either normal or with hyperplastic polyps, dysplastic (adenomas) or neoplastic (adenocarcinomas) colonic lesions, and the viral shedding through urine, in an adult Portuguese population; and b) the potential association between the presence of the virus in the colon and the lesions development, severity and grade, using a panel of lesions with different grades of hyperplasia/dysplasia/neoplasia.

2.3. Patients and Methods

2.3.1. Clinical Samples

All the individuals included in this study were selected exclusively when having medical indication to perform colonoscopy and biopsy (diagnostic and/or therapeutic), for evaluation of colorectal disorders (Appendix A.I), or surgery for colorectal tumour resection. During the procedure, we gathered the relevant clinical data (age, gender, previous clinical history, etc.) from each individual selected for the study (Appendix A.II). The harvesting and handling of all clinical samples were

performed in the Gastroenterology Department of the Hospitais da Universidade de Coimbra, EPE (HUC) and in the Surgery Department of the Centro Hospitalar de Coimbra, EPE (CHC), after approval by the respective Ethic Committees and according to its guidelines and protocols (reference CES009, 2008 January 17). All patients gave their written informed consent before admission to the study (Appendix A.III).

From 200 volunteers, 830 human clinical specimens were harvested, 740 corresponding to colorectal mucosa samples (with approximately 2x2mm), harvested under colonoscopy or surgery, and 90 corresponding to 20-50ml of urine (Appendix A.IV).

The selection between the study group and the control group was made during colonoscopy: if at least one colorectal hyperplastic/dysplastic/neoplastic lesion was present the individual was included in the study group (patients) and if no lesion was found then the individual entered the control group (controls). All the individuals submitted to colorectal surgery for tumour resection were included in the study group.

One hundred volunteers were classified as controls (61 males and 59 females), showing no clinical history of polyps or oncological colorectal lesions (hyperplastic polyps, adenomas or adenocarcinomas). The average age was 63 years \pm 13.37 (23 to 93 years old). Furthermore, one hundred patients (67 males and 33 females) were selected for the study group, showing colorectal hyperplastic/dysplastic/neoplastic lesions (at least one hyperplastic polyp, adenoma or adenocarcinoma) during colonoscopy or surgery, with an average age of 66 years \pm 8.73 (32 to 90 years old).

In the control group (n=100), 2 biopsy sections and 1 urine sample *per* individual were harvested, in a total of 200 biopsy specimens of normal colorectal mucosa and 30 urine samples. From 70 controls it was not possible to collect urine samples due to non-compliance or physiological inability.

In the study group (n=100), 7 specimens *per* patient were harvested: a) 2 from hyperplastic polyps/adenomas/adenocarcinomas (n=140); b) 2 from each normal colorectal mucosa, both adjacent to the lesions (within a perimeter of 5mm) (n=200) and non-adjacent to the lesions (from the proximal colon when hyperplastic polyps/adenomas/adenocarcinomas were in the distal colon, and *vice-versa*) (n=200), in a total of 540 colorectal mucosa specimens; and c) 1 urine sample (n=60). From 60 patients only 1 biopsy sample, corresponding to hyperplastic polyps/adenomas/adenocarcinomas, was harvested during colonoscopy due to the small size of the lesions. From 40 patients we were not able to collect the urine sample due to specific clinical conditions.

Each colorectal mucosa sample was preserved in an 1.5ml eppendorf tube with 200 μ l of RNAlater stabilization reagent (Applied, Carlsbad, California) (for posterior studies using RNA), and kept at -80°C. The urine samples were preserved in a 60ml sterilized container and kept at 4-6°C, until experimental procedures. In this study we used only one specimen of each type of the colorectal

mucosa samples and a 15ml aliquot of the urine samples from each individual. The remaining specimens were preserved at -80°C for posterior handling.

The anatomico-pathological diagnosis and staging were carried out in matched pairs of clinical samples, sent to the Anatomico-Pathology Departments of HUC and CHC, according to the World Health Organization (WHO) Classification of Tumours of the Gastrointestinal Tract. This procedure allowed establishing the differential diagnosis of colorectal hyperplastic polyps, tubular, tubulovillous and villous adenomas, and adenocarcinomas.

2.3.2. Nucleic Acids Extraction

Total genomic DNA extraction and purification, from colorectal mucosa specimens kept in RNAlater stabilization reagent, was performed by using the InvisorbSpin Tissue Mini Kit (Invitek, Berlin, Germany) according to manufacturer's protocol.

For total DNA extraction from urine samples we adapted and optimized an experimental protocol, previously described by Chang *et al*[30] for detection of human papillomavirus (HPV) in urine samples. Urine samples were homogenized and aliquoted into 15ml falcon tubes and only 10ml of each sample were used in this experimental procedure. The samples were centrifuged at 5,500rpm (5444xg), in a refrigerated centrifuge (3-16K, Sigma, Madrid, Spain), at 4°C for 30'.

After centrifugation, the supernatant was discarded; the pellet was washed with 1ml of sterile $1\times$ PBS (137mM NaCl, 2.7mM KCl, 10mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.4) and again centrifuged for 10'. In the end, the washing solution was discarded and the pellet re-suspended with 500 μl of 1x digestion buffer (100mM Tris-HCL, 10mM EDTA, pH 8.0), 50 μl of Proteinase K (500 $\mu\text{g}/\text{ml}$) (5 Prime, Hamburg, Germany) and mixed in the vortex for a few seconds. The solution was then incubated at 50°C over-night (O/N), for complete digestion, and Proteinase K was finally inactivated at 95°C for 10'. The cellular debris was precipitated by centrifugation for 5' and the supernatant was collected in a fresh 1.5ml eppendorf tube.

For total DNA extraction, the samples were then treated according to a phenol/chloroform/isoamyl alcohol (25:24:1(v/v/v)) (Applied Biosystems, Weiterstadt, Germany) protocol, consisting of successive washes with this solution. We added 500 μl to the lysate, gently inverted the tube for 2' and centrifuged the mix at 13000rpm (10000g) for 10' at room temperature (RT). The aqueous supernatant, containing the nucleic acids, was collected in a clean eppendorf tube and the same procedure was repeated twice. A final wash with 500 μl of chloroform pro-analysis (Merck, Darmstadt, Germany) and an additional centrifugation, for 10', allowed the elimination of phenol residues from the nucleic acid solution.

To retrieve the suspended DNA, we added 50 μl of 3M sodium acetate (Merck, Darmstadt, Germany), 500 μl of 95% ethanol (Merck, Darmstadt, Germany) and centrifuged at 13000 rpm for 30' at RT. After discarding the supernatant, the precipitate was then washed twice with 500 μl of 75%

ethanol and left to dry O/N at RT. DNA was finally re-suspended with 50µl milli-Q water and stored at -20°C until manipulation.

Additionally, we evaluated DNA yield and purity by total DNA quantification, in the NanoDrop (Thermo Scientific, Wilmington, USA), for all the urine and colorectal mucosa samples. The urine samples treated with our adapted experimental approach, despite its extended procedure, presented higher DNA concentrations, ranging from 10.00 to 78.00ng/µl.

2.3.3. Detection of JCV DNA Sequences

In order to detect JCV DNA sequences in all the clinical samples, corresponding to colorectal mucosa and urine specimens, we performed Nested-PCR with two specifically designed set of primers (prepared by StabVida, Caparica, Portugal), complementary to a consensus sequence within the viral gene encoding the small t-antigen oncoprotein (t-Ag) of the JCV-Mad-1 strain[31].

The first Nested-PCR reaction was carried out with a PCR mixture of 50µl, containing 1× PCR incomplete buffer (50mM KCl, 10mM Tris-HCl) (Bioron, Ludwigshafen, Germany), 2mM of Mg²⁺ (Bioron, Ludwigshafen, Germany), 200µM of dNTPs (Bioron, Ludwigshafen, Germany), 20pmol of the external primer set (JCTR1F 5'-cttgggtaagtacacacca-3' and JCTR2R 5'-atgcaaagaactccaccctg-3'), 2.5 units of Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 50ng of DNA obtained from each clinical sample and Milli-Q water to complete the final volume. The reactions were then submitted to thermal cycling: a hot-start (94°C for 10''), followed by 40 denaturing cycles at 94°C for 30'', annealing at 58°C for 30'' and extension at 72°C for 30'', with a final extension step at 72°C for 10'.

The second Nested-PCR reaction was performed in the same conditions, previously described, but using 5µl of the first PCR products as target DNA template, 20pmol of the internal primer set (JCTR3F 5'-tccacacaagtgggctgctt-3' and JCTR4R 5'-ggtggggacgaagacaagat-3') and an annealing temperature of 60°C.

In all Nested-PCR amplifications there were both blank and positive controls. As blank control, a similar mixture was prepared with no DNA (replaced by the corresponding volume of milli-Q water) and as positive control we used the plasmid pBR322 – BamHI with the JCV-Mad-1 genome, kindly provided by Kamel Khalili MD PhD (Neurovirology and Cancer Biology Department, Temple University, Philadelphia – USA).

Then, 10µl of each Nested-PCR product was resolved by horizontal electrophoresis on 2% agarose gels (Bio-Rad, Hercules, California), prepared with 1xTAE (40mM Tris, 20mM acetic acid, and 1mM EDTA, pH 8.0), stained with 1:20 (v/v) ethidium bromide (Merck, Germany) and visualized under UV light. The JCV DNA sequences size of successfully amplified samples by Nested-PCR was identified by comparison with the fragments of a known molecular weight marker, 100 b.p. ladder (Bioron, Ludwigshafen, Germany) (Figure 2.1).

2.3.4. Statistical Analysis

Statistical analysis was performed using the Graph-Pad Prism Version 6.0 (GraphPad Software, Inc., San Diego, California). Categorical data were analysed with the Chi-Squared or Fisher's Exact Test when appropriated. Differences were considered significant when $p < 0.05$. Results are presented in terms of relative frequencies.

When comparing the study group and the control group, and between samples from each group, we have used a rough estimate of risk, the Ratio of Cross-Products or Odds Ratio (OD), with intervals for a statistical confidence (CI) of 95%, and the strength of association was performed with the Cramer's Formula.

2.4. Results

Using the Nested-PCR assay, with our novel primer set targeted to a conserved consensus region of the JCV t-Ag gene, we were able to detect viral nucleotide sequences in 169 of the 200 studied individuals (84.5%).

In the study group, JCV DNA sequences were present in 90 of the 100 (90%) colorectal lesions: in 23 of 26 (89%) hyperplastic polyps, 39 of 42 (93%) tubular adenomas, 7 of 7 (100%) villous adenomas, 9 of 11 (82%) tubulovillous adenomas and 12 of 14 (86%) adenocarcinomas (Table 2.1). JCV DNA sequences were also detected in 48 of the 100 (48%) normal adjacent mucosa samples, all of them adjacent to positive colorectal lesions, and in 41 of the 100 (41%) normal non-adjacent mucosa samples (Figure 2.2A). In this group, the presence of JCV DNA sequences in hyperplastic polyps/adenomas/adenocarcinomas was significantly higher compared with the other biological specimens (Table 2.2).

No JCV DNA sequences were detected in 60 of the 100 (60%) normal mucosa samples of the control group (Figure 2.2B), and the estimated risk was not significantly different from the corresponding colorectal samples of the study group (Figure 2.2C). We also detected JCV DNA sequences in 39 of the 90 (43%) collected urine samples, without statistically significant differences between estimates of risk of viral shedding in the urine of both groups (Figure 2.2D). The registered frequencies were 48% and 37%, for patients and controls, respectively.

In the study group, if we consider the characteristics of the colorectal lesions, we found that the presence of JC virus was significantly higher in adenomas with low-grade of dysplasia, being this the predominant grade in the studied patients (Table 2.1).

Finally, in the control group, all the individuals that showed viral shedding in urine were also positive for the viral genome in the normal colorectal mucosa (Figure 2.3A), whereas, in the study group, the most common phenotype, considering the viral presence/absence, was that with positivity in the colorectal lesion with no JCV DNA sequences in the match pairs of normal mucosa (Figure 2.3B).

Furthermore, when there was no positivity in the colorectal lesions, the normal adjacent and non-adjacent mucosa were also negative (Table 2.3).

On comparing patients and controls, JCV DNA was preferentially present in hyperplastic polyps/adenomas/adenocarcinomas than in normal adjacent (OD 13.58), non-adjacent (OD 9.88) mucosa or in urine (OD 9.66). In the study group, the presence of JCV DNA sequences in hyperplastic polyps/adenomas/adenocarcinomas is significantly associated (OD 33.67; CI - 1.90-595.21) with its presence in one of the matched pairs of normal mucosa or in both. When this estimate of risk is transformed in a strength of association, using the Cramer's formula, we obtain a $V=0.94$, showing that the presence of JCV DNA in colorectal lesions is 94% associated with its presence, at least, in one or in both matched pairs of normal mucosa (adjacent and non-adjacent).

Whenever no viral genome is present in hyperplastic polyps/adenomas/adenocarcinomas, the matched pairs of normal adjacent and non-adjacent mucosa were also negative, reinforcing that the presence of JCV DNA in colorectal lesions is associated with its presence in any of the normal mucosa or in both.

2.5. Discussion

Despite the high CRC incidence and mortality rates in Portugal[29], this is the first report concerning the presence of the JC virus in the gastrointestinal and urinary tracts of a standard adult Portuguese population, and a pioneer study on its occurrence in the normal and abnormal colorectal mucosa. Generically, in our population sub-set, the prevalence rate for JCV infection does not significantly differ from the serological studies stating that JC virus antibodies are detectable in 60-80% of the world adult populations[32,33].

Probably, in a geographical, genetic and technically-dependent manner, JCV has been detected in the human normal gastrointestinal mucosa (prevalence rates – from 30 to 89%)[27,34,35], colorectal adenomas (prevalence rates – from 42 to 88%)[35,36] and in CRC (prevalence rates – from 61 to 89%)[27,35,37]. Others found JCV nucleotide sequences in 28-80% of adenocarcinoma lesions, but less frequently in benign adenomas and not in the normal surrounding tissue, even in the normal adjacent colorectal mucosa[36,38,39]. On excluding the results for the normal colorectal mucosa, our findings for adenomas, adenocarcinomas and urine (Figure 2.2) are quite similar to the highest prevalence rates described in the previously referred reports.

Considering the published data for the different stages of colorectal lesions[34], we found comparable relative frequencies of JCV DNA sequences in hyperplastic polyps, tubular adenomas, villous adenomas, tubulovillous adenomas and CRC (Table 2.1). The higher presence of JCV DNA in colorectal lesions *versus* normal mucosa, either adjacent or non-adjacent, cannot be explained by

occurring through blood contamination, since we assured that colorectal samples harvesting was cautiously performed in the apical/distal portion of the lesions. Instead, we assume that the high prevalence of JVC DNA sequences in colorectal lesions may be due to a cellular environment prone to viral replication and that it is not related with the viral carcinogenic potential, since the presence in hyperplastic polyps is quite similar to the presence in adenomas. In fact, our data suggest that JCV presence may be closely associated with the lesion itself rather than with its type, grade or severity. On the other hand, the absence or scarcity of the virus does not necessarily preclude its potential role in host-cell transformation, because it can be silenced or its genome can be lost during tumour progression (“hit-and-run” transformation)[40].

On the contrary, some authors were not able to demonstrate the JCV association with colorectal lesions: Hernández Losa *et al*[41] failed to detect the viral sequences by Nested-PCR, using a set of primers that amplified a conserved region within the viral gene that encodes the large T-antigen, and they were unable to stain the T-Ag protein, by immuno-histochemistry, either in colon adenocarcinomas or in the normal colorectal mucosa. They highlighted that, probably, the epidemiology of polyomaviruses in the United States should be different from the one in Europe. However, our results, and those from similar experimental approaches[35], differ from these observations of Losa and colleagues, possibly due to the set of primers we designed, targeted to a consensus sequence within the viral gene that encodes the small t-antigen (t-Ag) oncoprotein of the JCV Mad-1 strain.

Some may ask why we choose to look at viral t-Ag DNA and not T-Ag. It is opportune to refer that most detection studies by other groups have been designed targeting the JCV large-T antigen, which is common to other viruses, such as BK and SV40, to the point that these viruses cannot be discriminated by antibodies against T-Ag due to cross-reaction. In addition, a similar problem might occur in PCR if it is not nested. This is a reasonable explanation for the wide variation among different studies targeting T-Ag, particularly in earlier studies on JCV prevalence. Attending these findings, and considering that the restriction of the pathogenic role to a single viral protein (T-Ag) is an oversimplification, we chose to use an alternative viral sequence as target.

Although our data cannot support or exclude the following considerations, some authors explain this variability in JCV detection, in an infected colon, through the viral integration in the genome of some host-cells, with partial loss of JCV DNA. This event may have, in fact, a pathologic role in cancer development and can trigger additional processes leading to cancer progression by the selection of a cell subpopulation (Figure 1). Furthermore, the inflammatory infiltration itself, caused by a local chronic infection in the colon, can also contribute to the selection and expansion of a tumour-prone cell[1,34,38,42,43].

Concerning the viral shedding in urine, some studies showed that, in cancer patients, viruria can be detected in up to 45% of the cases, even when JCV DNA could not be detected in adenoma/adenocarcinoma tissues[44]. Considering just the Portuguese population, only two related studies characterize the molecular epidemiology of the urinary excretion of JCV[45,46] both in healthy individuals (with excretion rates of 24% and 33%, respectively) and in HIV patients (with excretion rates of 51%) and that it is highly variable between genders and influenced by age[46]. Our findings point to a higher frequency of the virus in urine of both control and study groups, either dependent on the reduced number of specimens, the optimized DNA extraction protocol or the higher sensitivity of our set of primers.

To our knowledge, a critical issue in order to determine if JCV contributes to CRC is, indeed, its presence in the normal mucosa surrounding the colonic lesions, either adjacent or non-adjacent. If we presume that JCV infection plays no role in the colorectal carcinogenesis, then the values for its detection should be similar, regardless of the mucosa type (normal/abnormal), which is not the case of the results from our study. But if we consider that JCV presence is able to facilitate tumour development, DNA sequences should be detected at significant levels in the tumour tissue.

Our analysis of the comparison between colonic lesions and the surrounding normal mucosa showed that the frequency is, in fact, higher in the tumour tissue than in the matched pairs of normal mucosa. Furthermore, in this context it is important to emphasize that there were no significant differences between the values observed for the normal mucosa surrounding the lesions versus the control mucosa, which are in consonance with the lack of significance between the two groups of patients concerning to the viral urinary secretion.

These data show us the epidemiological extent of the JCV infection in the normal Portuguese population, detectable in both normal colorectal mucosa and urine. But in a subgroup of patients with colorectal lesions, the higher prevalence of JCV DNA suggests that a biological selection of the JCV infected cells occurs during tumourigenesis.

2.6. Conclusions

The role of JC virus in carcinogenesis is still an open issue in the pathogenesis of colorectal cancer. Even with substantial discrepancies, probably dependent of variables such as geography, genetic background and technical approach, JCV has been successfully detected in several studies concerning to its association with CRC development.

The high prevalence rates, found for the presence of JCV DNA in the colorectal mucosa and urine, seems to be based on the our experimental approach and the selected set of primers, with newly designed oligonucleotide sequences complementary to the viral gene encoding t-Ag. These primers are

probably more accurate for the detection of JCV genome, either in colorectal mucosa or in urine samples, than those targeted for large T-antigen, when there is partial DNA loss due to vital integration, with consequent lack of target for primer amplification. The integration of viral DNA in viruses-associated tumours is a well known phenomenon, namely for viruses such as HPV and HBV.

The results of this study allowed us to consider that the presence of JCV DNA in colorectal lesions is an early and sustained event during tumour progression, unrelated with the lesion type, grade or severity, and that JCV may have a specific tropism for CRC and colorectal polyps. This observation may reflect a condition where the transformed colorectal epithelial cells offer a more permissive environment for viral DNA replication, being effective even since the hyperplastic polyps not prone to carcinogenesis. In fact, all together, our findings suggest that JC polyomavirus infection may precede the colorectal lesion occurrence, acting as an additional risk factor, or just be related with its progression, acting as a carcinogenic enhancer.

Furthermore, we propose the epidemiological extent of the JCV infection in the gastrointestinal and urinary tracts of the normal Portuguese population, and a biological selection of JCV infected cells during tumourigenesis in individuals with colorectal lesions.

On the other hand, the inflammatory infiltration caused by the local chronic infection of the colorectal mucosa can contribute, itself, to the selection and expansion of tumour-prone cells. In this context, JCV may assume an important role as a co-factor in neoplastic transformation and development of colonic tumours. This is an issue that has not been explored in the context of gastrointestinal JCV infection and which deserves further attention, particularly if considering that chronic inflammation is a risk factor in colorectal cancer.

Finally, we consider that the short-term planning of strategies to reduce the burden of JCV in the population might contribute to reduce the incidence of colorectal cancer, similarly to other human cancer associated to infectious agents. In addition, the local inflammatory reaction in response to chronic infection, even if mild, might also be an important factor to facilitate tumour development, and it can also be a target in order to reduce its risk.

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2.8. Web Site Support

- The National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>
- Cancer Research UK: <http://www.cancerresearchuk.org>
- PLOS Pathogens: <http://www.plospathogens.org>
- ViroBLAST: <http://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php>

2.9. Figures and Tables

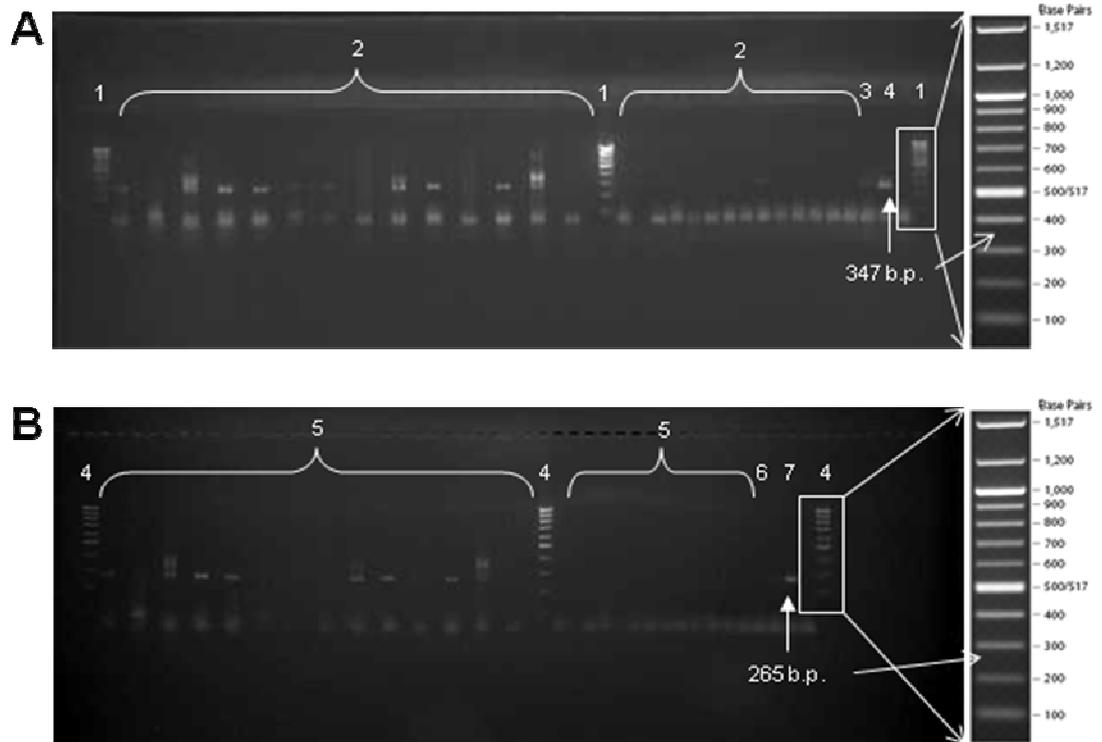


FIGURE 2.1

A: 1st Nested-PCR reaction using the external set of primers; 1 – 100 b.p. ladder; 2 – PCR amplimers obtained from DNA extracted from colorectal mucosa samples ; 3 – Blank control; 4 – Positive control (PCR amplimer obtained from the plasmid pBR322 – BamHI with JCV-Mad-1 genome);

B: 2nd Nested-PCR reaction using the internal set of primers; 4 – 100 b.p. ladder; 5 – PCR amplimers obtained from DNA extracted from colorectal mucosa samples ; 6 – Blank control; 7 – Positive control (PCR amplimer obtained from the plasmid pBR322 – BamHI with the JCV-Mad-1 genome).

TABLE 2.1. Relationship between JCV DNA presence/absence and colorectal lesions characteristics.

n=100	JCV		
	Number of Patients	Presence	Absence
1. Hyperplastic polyps	26	23	3
1.1. Male/Female ratio	18/8	16/6	2/2
1.2. Location			
1.2.1. Rectum	11	11	0
1.2.2. Sigmoid colon	15	12	3
2. Adenomas	60	55	5
2.1. Male/Female ratio	41/19	38/17	3/2
2.2 Histological type			
2.2.1. Villous	7	7	0
2.2.2. Tubular	42	39	3
2.2.3. Tubulovillous	11	9	2
2.3. Grade of dysplasia			
2.3.1. High-grade	1	1	0
2.3.2. Low-grade	59	57	2
2.4. Location			
2.4.1. Right colon	10	10	0
2.4.2. Left colon	50	45	5
3. Adenocarcinomas	14	12	2
3.1. Male/Female ratio	8/6	7/5	1/1
3.2. Dukes stage			
3.2.1. A	3	2	1
3.2.2. B	2	2	0
3.2.3. C	6	5	1
3.2.4. D	3	3	0
3.3. Degree of differentiation			
3.3.1. Good	9	8	1
3.3.2. Moderate	5	4	1
3.3.3. Poor	0	0	0
3.4. Location			
3.4.1. Right colon	2	2	0
3.4.2. Left colon	12	10	2

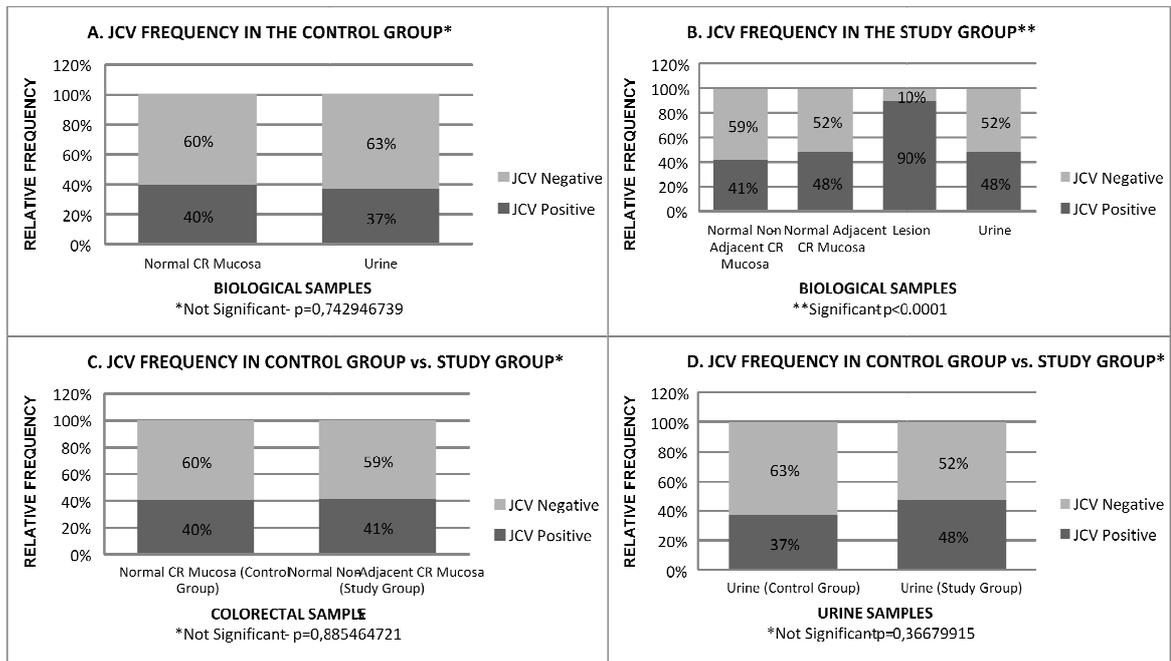


FIGURE 2.2

A: Relative frequency in the samples of the control group (with no colorectal lesions), for the presence (JCV positive) and absence (JCV negative) of JCV DNA sequences; studied samples correspond to normal colorectal mucosa and urine; the percentages refer to relative frequency;

B: Relative frequency in the samples of the study group (with, at least, one colorectal hyperplastic polyp, adenoma or adenocarcinoma), for the presence (JCV positive) and absence (JCV negative) of JCV DNA sequences; studied samples correspond to colorectal lesions, normal adjacent and non-adjacent mucosa and urine; the percentages refer to relative frequency;

C: Comparison between normal colorectal mucosa samples from the study group and from the control group, for the presence (JCV positive) and absence (JCV negative) of JCV DNA sequences;

D: Comparison between urine samples from the study group and from the control group, for the presence (JCV positive) and absence (JCV negative) of JCV DNA sequences.

TABLE 2.2. Risk estimate (Odds Ratio=OD) and Confidence Intervals (CI) for the presence of JCV DNA sequences in colorectal lesions (hyperplastic polyps/adenomas/adenocarcinomas) *versus* the other biological specimens from the study group.

	Odds Ratio (OD)	Confidence intervals (CI-95%)	Significance	<i>p Value</i>
Colorectal Lesions vs. Normal adjacent mucosa	9.75	4.550-20.90	****	<i>p</i> <0.0001
Colorectal Lesions vs. Normal non-adjacent mucosa	12.95	6.024-27.84	****	<i>p</i> <0.0001
Colorectal Lesions vs. Urine	10.29	4.497-23.52	****	<i>p</i> <0.0001

OD=Odds Ratio

CI=Confidence Intervals

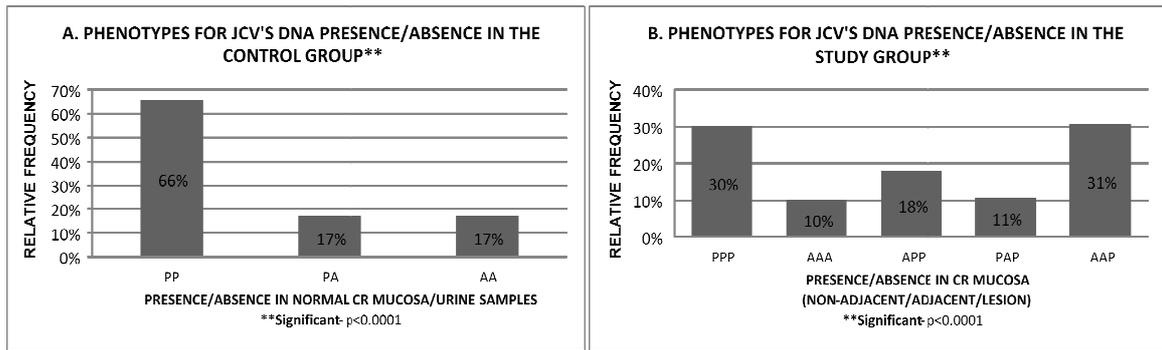


FIGURE 2.3

Different combinations (phenotypes) of the sample type for the presence (P) and absence (A) of JCV DNA sequences:

A: in the control group (studied samples correspond to normal colorectal mucosa and urine) and

B: in the study group (studied samples correspond to normal non-adjacent mucosa, normal adjacent mucosa and colorectal lesions).

P=Presence of JCV DNA

A=Absence of JCV DNA

TABLE 2.3. Absolute frequency of the presence (P)/absence (A) of JCV DNA sequences in the observed combinations (phenotypes) of the sample types (normal non-adjacent mucosa, normal adjacent mucosa, lesions and urine).

	Phenotypes				
	(P)/(P)	(P)/(A)	(A)/(P)	(A)/(A)	
Control Group					
(Normal Colorectal Mucosa/Urine)					
n=30	20	5	0	5	
<i>p Value (X²)</i>	<0.0001 (22.50)*				
Study Group	(P)/(P)/(P)	(A)/(A)/(A)	(A)/(P)/(P)	(A)/(A)/(P)	(P)/(A)/(P)
(Normal Non-Adjacent Mucosa/Normal Adjacent Mucosa/Lesions)					
n=100	30	10	18	31	11
<i>p Value (X²)</i>	<0.0001 (25.38)*				

*Significant

P=Presense

A=Absence

2.10. Appendix A

2.10.1. Appendix A.I

CRITÉRIOS DE INCLUSÃO E EXCLUSÃO

COLONOSCOPIA E BIÓPSIA COLORRECTAL

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

1. Critérios Gerais de Inclusão no Protocolo de Estudo

Deverão ser incluídos no protocolo de estudo os indivíduos que possuam uma das seguintes características:

1.1 - Doentes com indicação clínica de realização de colonoscopia e eventual biópsia diagnóstica/terapêutica, numa das seguintes situações:

- Sangramento digestivo;
- Diarreia crónica;
- Doença inflamatória crónica do cólon;
- Estenoses cólicas;
- Parasitoses;
- Pólipos do cólon e/ou reto identificados durante o exame;
- Tumores colorrectais.

1.2 - Doentes com indicação de colheita de biópsia:

- Em qualquer uma das situações anteriores, desde que haja indicação de biópsia;
- Em qualquer outra situação clínica que necessite de biópsia diagnóstica (ex.: fasciolíase).

Nota: Os exames deverão ser indicados para avaliação diagnóstica elegível de quadros clínicos sugestivos de alterações colorrectais e no seguimento de tratamentos clínicos e cirúrgicos em doentes em ambulatório e internados.

1.3 - Doentes com patologias que constituam fatores de risco de desenvolvimento de pólipos colorrectais:

- Doentes com acromegalia, já operados ou não;
- Doentes com bacteriemia por *Streptococcus bovis*;
- Doentes com cancro da mama;
- Doentes colecistectomizados;
- Doentes com ureterosigmoidostomia.

1.4 - Doentes de risco de endocardite infecciosa ou portadores de prótese valvar cardíaca desde que seja feita antibioticoprofilaxia (ex.: associação de ampicilina e gentamicina);

1.5 - Doentes em uso de terapia anticoagulante desde que sujeitos a suspensão temporária da medicação: doentes em uso de anti-agregantes plaquetares ou anti-inflamatórios não-esteróides desde que o seu uso seja suspenso nos 7 dias anteriores à data da realização do exame.

2. Critérios Gerais de Exclusão no Protocolo de Estudo

Não deverão ser incluídos no protocolo de estudo os indivíduos com uma das seguintes condições:

2.1 - Menores de 18 anos;

2.2 - Grávidas (beta-HCG positivo);

2.3 - Doentes com sintomatologia e/ou patologia colo-rectal, sem indicação de biópsia;

2.4 - Peritonite;

2.5 - Distúrbios de coagulação;

2.6 - Imunocomprometidos (ex.: diabetes melitus, insuficiência renal, HIV, radioterapia, corticoterapia, outros imunossupressores). Nestes casos poderão ser indicados alguns exames para avaliar o doente antes de realizar o exame. A contagem de linfócitos T CD4+ deve ser superior a 200/mm³, ou quando abaixo desse número, os leucócitos devem ser superiores a 3.000/mm³;¹⁰ as plaquetas devem estar acima de 50.000/mm³; a hemoglobina superior a 10 g/dl e o tempo de protrombina maior que 60%;

2.7 - Dispneia;

2.8 - Cardiopatia avançada;

2.9 - Doentes sem autonomia;

2.10 - Doentes não colaborantes;

2.11 - Doentes que apresentem mau estado geral;

2.12 - A não concordância com os termos do Consentimento Livre Esclarecido;

2.13 - Síndromas hereditários: Polipose Adenomatosa Familiar e Síndrome de Lynch;

2.14 - Indicação para realização urgente de colonoscopia (ex.: quadro oclusivo, hemorragia digestiva com repercussão hemodinâmica).

Nota: Entre os riscos acrescidos à realização de biópsia conta-se a bacteriemia pós-colonosopia. Na população em geral a incidência está em torno de 0% a 5%, independente de biópsia ou polipectomia.

2.10.2. Appendix A.II

FORMULÁRIO DE RECOLHA DE DADOS

PROJETO POLIOMAVÍRUS

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia



HOSPITAL: _____

SERVIÇO: _____

N.º REGISTO/ENTRADA: _____ / _____

DATA: ____ / ____ / ____

DOENTE A INCLUIR NO GRUPO: _____

DADOS PESSOAIS					
APELIDO			SEXO:	M	<input type="checkbox"/>
NOME				F	<input type="checkbox"/>
DATA DE NASCIMENTO	____/____/____				
ORIGEM GEOGRÁFICA:			CONCELHO		
			DISTRITO		
MORADA ACTUAL					
CÓDIGO POSTAL	____ - ____	LOCALIDADE			
AGREGADO FAMILIAR	____ pess.	ESCOLARIDADE			
	PROFISSÃO				
HÁBITOS TABÁGICOS	N	<input type="checkbox"/>			
	S	<input type="checkbox"/>	CONSUMO DIÁRIO:	MENOS DE 1 MAÇO	
				1-2 MAÇOS	
				MAIS DE 2 MAÇOS	
HÁBITOS ALCOÓLICOS	N	<input type="checkbox"/>			
	S	<input type="checkbox"/>			

DADOS CLÍNICOS			
N.º PROCESSO CLÍNICO		DATA DE ENTRADA	___/___/_____
MÉDICO ASSISTENTE		N.º MECANOGRÁFICO	
SERVIÇO			
DIAGNÓSTICO CLÍNICO			
IDADE AQUANDO DO DIAGNÓSTICO			_____ anos
TIPO DE INTERVENÇÃO:	BIÓPSIA DIAGNÓSTICA		<input type="checkbox"/>
	BIÓPSIA TERAPÉUTICA		<input type="checkbox"/>
	CIRURGIA DE RESSECÇÃO TUMORAL		<input type="checkbox"/>
	COLECTOMIA		<input type="checkbox"/>
	OUTRA		QUAL:
HISTÓRIA CLÍNICA ANTERIOR	<hr/> <hr/> <hr/>		
HISTÓRIA FAMILIAR	<hr/> <hr/> <hr/>		

DADOS DA COLHEITA			
DATA DA COLHEITA	__/__/____	HORA DA COLHEITA	__:__
MÉDICO REQUISITANTE		N.º MECANOGRÁFICO	
SERVIÇO			
TIPO DE MATERIAL BIOLÓGICO:	SANGUE PERIFÉRICO		
	URINA		
	ESFREGAÇO BUCAL		
	TECIDO FRESCO		
	TECIDO PARAFINADO		
	TECIDO CONGELADO		
	OUTRO		QUAL:
N.º DE AMOSTRAS COLHIDAS		DESIGNAÇÃO	1 -
			2 -
			3 -
			4 -
DESCRIÇÃO DAS AMOSTRAS	1 -		
	2 -		
	3 -		
	4 -		
DESIGNAÇÃO CORRESPONDENTE DA AMOSTRA ENVIADA À ANATOMIA-PATOLÓGICA	1 -		
	2 -		
	3 -		
	4 -		
DIAGNÓSTICO ANATOMO-PATOLÓGICO	_____		

2.10.3. Appendix A.III

FORMULÁRIO DE CONSENTIMENTO LIVRE ESCLARECIDO

(De acordo com a versão oficial de 2004 da Declaração de Helsínquia sobre os Princípios Éticos de Investigação Médica Em Humanos e com a Convenção para a Proteção dos Direitos do Homem e da Dignidade do Ser Humano face às Aplicações da Biologia e da Medicina ratificada pela Resolução da Assembleia da República n.º 1/2001, de 3 de Janeiro)

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

Para doentes com patologia colorrectal a serem submetidos a colonoscopia e eventual biópsia diagnóstica/terapêutica

I. INTRODUÇÃO

Está a ser convidado(a) para fazer parte de um estudo de investigação. Antes de decidir fazer parte deste estudo, é necessário que compreenda os riscos e os benefícios envolvidos. Este formulário de consentimento fornece informações sobre o estudo de investigação. Um membro do grupo de investigação, envolvido neste estudo, estará sempre ao seu dispor para responder às suas perguntas e fornecer explicações adicionais sempre que as solicitar. Se concordar em fazer parte deste estudo de investigação, ser-lhe-á pedido que assine este formulário de consentimento. Este processo é conhecido por consentimento livre esclarecido ou com conhecimento de causa. A decisão é sua, sendo livre de optar entre fazer ou não parte deste estudo.

II. OBJETIVO

Este projeto de investigação tem como principais objetivos: o estabelecimento de uma correlação entre a infeção por poliomavírus neurotrópico humano (JCV) e o desenvolvimento de cancro colorrectal numa população Portuguesa; a avaliação dos respetivos fatores modificadores do risco e seu valor como fator de prognóstico; e a identificação dos mecanismos da carcinogénese colorrectal potencialmente mediada pelo vírus. Uma vez comprovada esta associação, será possível identificar os indivíduos de risco para o desenvolvimento de cancro colorrectal, ajustar a vigilância

clínica para um diagnóstico precoce e identificar, a longo prazo, possíveis alvos terapêuticos, quer virais, quer da célula hospedeira, com o intuito de controlar a patogenicidade do JCV.

Para avaliar a frequência da infeção pelo vírus JC em indivíduos portadores de cancro colorrectal, é necessário estudar mucosa colorrectal com lesão tumoral em vários estádios, mucosa colorrectal com lesão precursora de tumor, mucosa colorrectal com lesão não oncológica e mucosa normal adjacente às lesões. Pelo que fragmentos das amostras que poderão ser colhidas da sua mucosa colorrectal serão destinados a este projeto de investigação, desde que o consinta por escrito.

III. PROCEDIMENTOS

Ao concordar em participar, permite que, durante a realização da sua colonoscopia de diagnóstico ou de tratamento, se forem colhidas biópsias de mucosa do cólon, alguns fragmentos destas podem ser utilizados, no âmbito deste projeto, para estudos moleculares. A realização deste estudo não altera em nada o seu protocolo de tratamento e vigilância.

IV. POSSÍVEIS RISCOS

Os riscos associados às colheitas de mucosa colorrectal, por biópsia colonoscópica, são os mesmos associados às colheitas do material para diagnóstico ou tratamento da sua patologia, porque derivam dessas colheitas, sem que haja qualquer colheita adicional.

V. POSSÍVEIS BENEFÍCIOS

Com a sua participação neste estudo não existirá um benefício imediato para si, quer financeiro quer de qualquer outra natureza. No entanto, espera-se que o resultado deste projecto de investigação conduza à identificação de indivíduos de risco para o desenvolvimento de cancro colorrectal. Se for o seu caso, poderá beneficiar de uma adequada estratégia de vigilância e diagnóstico precoce. Mais se espera, com o seu contributo pessoal, melhorar o entendimento da carcinogénese colorrectal, mediada pelo vírus JC, e o respectivo tratamento.

VI. CONSENTIMENTO PARA UTILIZAÇÃO E ELIMINAÇÃO DAS AMOSTRAS

Através da assinatura deste formulário de consentimento, autoriza a utilização das suas amostras de mucosa colorrectal, colhidas por biópsia colonoscópica, para este estudo de investigação. Simultaneamente consente na eliminação, após manipulação, de qualquer tecido restante ou que não seja adequado ao estudo.

VII. CUSTOS

Não incorrerá em quaisquer despesas com a colheita das amostras, análises, testes e avaliações exigidas por este protocolo.

VIII. COMPENSAÇÕES

Não está disponível qualquer tipo de compensação financeira (nenhum tipo de pagamento) ou afim, para os participantes neste estudo.

IX. DIREITO A DESISTIR DO ESTUDO

A sua participação neste estudo de investigação é voluntária. Poderá decidir não começar ou cessar a sua participação neste estudo em qualquer altura que pretenda. Os cuidados que receber e as relações que estabelecer, com os prestadores de assistência médica dos Hospitais da Universidade de Coimbra, não serão afectados de maneira nenhuma. Ser-lhe-ão comunicadas quaisquer novas informações, sobre o estudo de investigação, que possam fazer com que mude de opinião sobre a sua participação. Deverá notificar um dos elementos do grupo de investigação, caso decida cessar a sua participação antes do tempo previsto.

X. CONFIDENCIALIDADE DOS RELATÓRIOS MÉDICOS E DA INVESTIGAÇÃO

Todos os seus dados pessoais e registos médicos, excepto no que se refere à equipa clínica e aos médicos integrantes do grupo de investigação, e todos os resultados do estudo de investigação, excepto no que se refere à equipa clínica e ao grupo de investigação, serão mantidos em sigilo, a menos que sejam exigidos por decisão judicial.

XI. PERGUNTAS

Se necessitar de algum esclarecimento relacionado com este trabalho de investigação ou se sentir algum efeito secundário ou lesão que possa estar relacionada com a sua participação no estudo, poderá contactar um dos elementos do grupo de investigação.

Os investigadores Mestre Tatiana Andrea Rasteiro Coelho e Prof. Doutor Luís Almeida serão responsáveis pelo esclarecimento, antes e após a colheita, de qualquer dúvida, pessoalmente, por telefone (239480054/916584854/912133241) ou por e-mail (tatiana.coelho@sapo.pt/lalmeida@ci.uc.pt).

XII. ASSINATURAS

Através da assinatura deste formulário de consentimento livre esclarecido, afirma que leu o seu conteúdo, que os objetivos e procedimentos deste estudo lhe foram explicados e que todas as suas perguntas foram devidamente e claramente respondidas. Não estará a abdicar de nenhum dos seus direitos legais, ao assinar este formulário. Adicionalmente afirma também que todas as informações prestadas por si, acerca dos antecedentes clínicos, são verdadeiras. Caso solicite, ser-lhe-á fornecida uma cópia deste consentimento.

Eu, _____,
portador(a) do B.I. n.º _____, emitido em ____ / ____ / ____ pelo Arquivo de Identificação de _____, e abaixo assinado(a), li e compreendi tudo o que consta neste formulário e esclareci todas as minhas dúvidas acerca da minha participação neste projecto de investigação, pelo que:

1 - Concordo em participar neste estudo, através da utilização da(s) minha(s) amostra(s) de mucosa colorrectal, colhida(s) aquando da realização de colonoscopia e biópsia para diagnóstico da minha patologia:

sim ()

não ()

2 – Visto que respondi sim, também concordo com o estudo e posterior divulgação científica dos dados obtidos a partir da(s) minha(s) amostra(s):

sim ()

não ()

3 - Concordo com a posterior divulgação científica das imagens obtidas a partir da(s) minha(s) amostra(s):

sim ()

não ()

4 – Pretendo tomar conhecimento dos resultados referidos nas duas alíneas anteriores:

sim ()

não ()

Coimbra, _____ de _____ de 20__

O participante

Coimbra, _____ de _____ de 20__

O seu representante autorizado

Relação com o participante: _____

Se o participante puder compreender o conteúdo mas não puder, por alguma razão, ler (ex.: cegueira, etc.) ou não puder assinar (ex.: incapacidade motora, etc.) o formulário de consentimento livre esclarecido, será necessária a presença de uma testemunha não relacionada com o estudo.

Coimbra, _____ de _____ de 20__

A testemunha

Coimbra, _____ de _____ de 20__

Nome e Assinatura dos Investigadores

FORMULÁRIO DE CONSENTIMENTO LIVRE ESCLARECIDO

(De acordo com a versão oficial de 2004 da Declaração de Helsínquia sobre os Princípios Éticos de Investigação Médica Em Humanos e com a Convenção para a Proteção dos Direitos do Homem e da Dignidade do Ser Humano face às Aplicações da Biologia e da Medicina ratificada pela Resolução da Assembleia da República n.º 1/2001, de 3 de Janeiro)

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

Para doentes a submeter a cirurgia colorrectal de ressecção tumoral

I. INTRODUÇÃO

Está a ser convidado(a) para fazer parte de um estudo de investigação. Antes de decidir fazer parte deste estudo, é necessário que compreenda os riscos e os benefícios envolvidos. Este formulário de consentimento fornece informações sobre o projeto de investigação. Um membro do grupo de investigação, envolvido neste estudo, estará sempre ao seu dispor para responder às suas perguntas e fornecer explicações adicionais sempre que as solicitar. Se concordar em fazer parte deste estudo de investigação, ser-lhe-á pedido que assine este formulário de consentimento. Este processo é conhecido por consentimento livre esclarecido ou com conhecimento de causa. A decisão é sua, sendo livre de optar entre fazer ou não parte deste estudo.

II. OBJECTIVOS

Este projeto de investigação tem como principais objetivos: o estabelecimento de uma correlação entre a infecção por poliomavírus neurotrópico humano (JCV) e o desenvolvimento de cancro colo-rectal numa população Portuguesa; a avaliação dos respectivos fatores modificadores do risco e seu valor como fator de prognóstico; e a identificação dos mecanismos da carcinogénese colo-rectal potencialmente mediada pelo vírus. Uma vez comprovada esta associação, será possível identificar os indivíduos de risco para o desenvolvimento de cancro colo-rectal, ajustar a vigilância clínica para um diagnóstico precoce e identificar, a longo prazo, possíveis alvos terapêuticos, quer virais, quer da célula hospedeira, com o intuito de controlar a patogenicidade do JCV.

Para avaliar a frequência da infecção pelo vírus JC em indivíduos portadores de cancro colo-rectal, é necessário estudar mucosa colo-rectal com lesão tumoral em vários estadios, mucosa colo-rectal com lesão precursora de tumor, mucosa colo-rectal com lesão não oncológica e mucosa normal adjacente às lesões. É para esta finalidade, se assim o consentir, que se destina o material biológico que for colhido durante a sua cirurgia.

III. PROCEDIMENTOS

Ao concordar em participar, permite que, durante o processamento da sua peça cirúrgica para tratamento e posterior diagnóstico, sejam colhidas amostras de mucosa do cólon para estudos moleculares. A realização deste estudo não altera em nada o seu protocolo de tratamento e vigilância, visto que não haverá colheita de amostras adicionais.

IV. POSSÍVEIS RISCOS

Esta colheita não incorre em quaisquer riscos adicionais, para além dos associados ao ato cirúrgico previsto.

V. POSSÍVEIS BENEFÍCIOS

Com a sua participação neste estudo não existirá um benefício imediato para si, quer financeiro quer de qualquer outra natureza. No entanto, espera-se que o resultado deste projeto de investigação conduza à identificação de indivíduos de risco para o desenvolvimento de cancro colo-rectal. Se for o seu caso, poderá beneficiar de uma adequada estratégia de vigilância e diagnóstico precoce. Mais se espera, com o seu contributo pessoal, melhorar o entendimento da carcinogénese colo-rectal, mediada pelo vírus JC, e o respectivo tratamento.

VI. CONSENTIMENTO PARA UTILIZAÇÃO E ELIMINAÇÃO DAS AMOSTRAS

Através da assinatura deste formulário de consentimento, autoriza a utilização das suas amostras de mucosa colo-rectal, colhidas da peça operatória após processamento para diagnóstico, neste estudo de investigação. Simultaneamente, consente na eliminação, após manipulação, de qualquer tecido restante ou que não seja adequado ao estudo.

VII. CUSTOS

Não incorrerá em quaisquer despesas com a colheita das amostras, análises, testes e avaliações exigidas por este protocolo.

VIII. COMPENSAÇÕES

Não está disponível qualquer tipo de compensação financeira (nenhum tipo de pagamento) ou afim, para os participantes neste estudo.

IX. DIREITO A DESISTIR DO ESTUDO

A sua participação neste estudo de investigação é voluntária. Poderá decidir não começar ou cessar a sua participação neste estudo em qualquer altura que pretenda. Os cuidados que receber e as relações que estabelecer, com os prestadores de assistência médica, não serão afectados de maneira nenhuma. Ser-lhe-ão comunicadas quaisquer novas informações, sobre o estudo de investigação, que possam fazer com que mude de opinião sobre a sua participação. Deverá notificar um dos elementos do grupo de investigação, caso decida cessar a sua participação antes do tempo previsto.

X. CONFIDENCIALIDADE DOS RELATÓRIOS MÉDICOS E DA INVESTIGAÇÃO

Todos os seus dados pessoais e registos médicos, excepto no que se refere à equipa clínica e aos médicos integrantes do grupo de investigação, e todos os resultados do estudo de investigação, exceto no que se refere à equipa clínica e ao grupo de investigação, serão mantidos em sigilo, a menos que sejam exigidos por decisão judicial.

XI. PERGUNTAS

Se necessitar de algum esclarecimento relacionado com este trabalho de investigação ou se sentir algum efeito secundário ou lesão que possa estar relacionada com a sua participação no estudo, poderá contactar um dos elementos do grupo de investigação.

Os investigadores Mestre Tatiana Andrea Rasteiro Coelho e Prof. Doutor Luís Almeida serão responsáveis pelo esclarecimento, antes e após a colheita, de qualquer dúvida, pessoalmente, por telefone (239480054/916584854/912133241) ou por e-mail (tatiana.coelho@sapo.pt / lalmeida@ci.uc.pt).

XII. ASSINATURAS

Através da assinatura deste formulário de consentimento livre esclarecido, afirma que leu o seu conteúdo, que os objetivos e procedimentos deste estudo lhe foram explicados e que todas as suas perguntas foram devidamente e claramente respondidas. Não estará a abdicar de nenhum dos seus direitos legais, ao assinar este formulário. Adicionalmente, afirma também que todas as informações prestadas por si, acerca dos antecedentes clínicos, são verdadeiras. Caso solicite, ser-lhe-á fornecida uma cópia deste consentimento.

Eu, _____,
portador(a) do B.I. n.º _____, emitido em ____ / ____ / ____ pelo Arquivo de Identificação de _____, e abaixo assinado(a), li e compreendi tudo o que consta neste formulário e esclareci todas as minhas dúvidas acerca da minha participação neste projeto de investigação, pelo que:

1 - Concordo em participar neste estudo, através da colheita de amostra(s) de mucosa colorectal da minha peça operatória, após manipulação e processamento para diagnóstico/tratamento da minha patologia:

sim ()

não ()

2 – Visto que respondi sim, também concordo com o estudo e posterior divulgação científica dos dados obtidos a partir da(s) minha(s) amostra(s):

sim ()

não ()

3 - Concordo com a posterior divulgação científica das imagens obtidas a partir da(s) minha(s) amostra(s):

sim ()

não ()

4 – Pretendo tomar conhecimento dos resultados referidos nas duas alíneas anteriores:

sim ()

não ()

Coimbra, _____ de _____ de 20__

O participante

Coimbra, _____ de _____ de 20__

O seu representante autorizado

Relação com o participante: _____

Se o participante puder compreender o conteúdo mas não puder, por alguma razão, ler (ex.: cegueira, etc.) ou não puder assinar (ex.: incapacidade motora, etc.) o formulário de consentimento livre esclarecido, será necessária a presença de uma testemunha não relacionada com o estudo.

Coimbra, _____ de _____ de 20__

A testemunha

Coimbra, _____ de _____ de 20__

Nome e Assinatura dos Investigadores

TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

(De acordo com a versão oficial de 2004 da Declaração de Helsínquia sobre os Princípios Éticos de Investigação Médica Em Humanos e com a Convenção para a Proteção dos Direitos do Homem e da Dignidade do Ser Humano face às Aplicações da Biologia e da Medicina ratificada pela Resolução da Assembleia da República n.º 1/2001, de 3 de Janeiro)

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Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

DECLARAÇÃO

Eu, _____,
portador(a) do B.I. n.º _____, emitido em ____ / ____ / ____ pelo Arquivo de Identificação de _____, e abaixo assinado(a), declaro que fui devidamente informado(a) e esclarecido(a) acerca do constante no consentimento livre esclarecido e que se resume a seguir:

1. - Um grupo de investigação da Faculdade de Medicina da Universidade de Coimbra encontra-se a desenvolver um projeto de investigação intitulado "Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a portuguese population and its association with cancer development", no qual se pretende detetar e identificar o poliomavírus neurotrópico humano JC, em amostras humanas da mucosa do cólon, visando o estudo da prevalência da infeção viral, numa população portuguesa, e a avaliação do risco de desenvolvimento de doenças oncológicas colorrectais.

2. - A equipa de investigadores, representada pelo Prof. Doutor Luís Manuel da Costa Marques de Almeida e pela Mestre Tatiana Andrea Rasteiro Coelho, é responsável pelo esclarecimento, antes e após a colheita, de qualquer dúvida, por telefone (912133241 ou 916584854) ou por e-mail (lalmeida@ci.uc.pt/tatiana.coelho@sapo.pt) e pelo levantamento e análise das amostras.

3. – *Os procedimentos a que serei submetido(a) para a obtenção destas amostras, os respectivos benefícios, riscos, complicações potenciais e alternativas decorrentes da minha participação foram-me descritos pelo médico especialista responsável pela colheita, o qual me garantiu que em nada colidem com todos os actos médicos que seriam praticados sem a existência desta investigação.*

Mais declaro que:

1. – *Me foi garantida a anulação do consentimento livre esclarecido, a qualquer momento, sem que disto decorra qualquer penalidade ou interrupção dos actos médicos referidos na alínea anterior.*

2. – *Me foi garantida a manutenção da total confidencialidade dos meus dados pessoais, em qualquer circunstância.*

3. – *Até ao momento, tive a oportunidade de colocar quaisquer questões, as quais foram pronta- e satisfatoriamente respondidas.*

4. – *Recebi explicações, li, compreendi e concordo com os itens acima referidos e que me foi dado a oportunidade de expressar sobre os pontos com os quais não concordasse.*

Após a leitura e a integral compreensão dos itens do consentimento livre esclarecido que me foi facultado, eu _____ declaro que:

1 - Concordo em participar nesta investigação, através da colheita de amostras adicionais da minha mucosa colorrectal aquando da realização da biópsia de diagnóstico e/ou terapêutica:

sim ()

não (.)

2 – Visto que respondi sim, também concordo com o estudo e posterior divulgação científica dos dados obtidos a partir da(s) minha(s) amostra(s):

sim ()

não ()

3 - *Concordo com a posterior divulgação científica das imagens obtidas a partir da(s) minha(s) amostra(s):*

sim ()

não ()

4 – *Pretendo tomar conhecimento dos resultados referidos nas duas alíneas anteriores:*

sim ()

não ()

Coimbra, _____ de _____ de 20__

O participante

Nome e Assinatura dos Investigadores

2.10.4. Appendix A.IV

PROTOCOLO DE COLHEITA DE AMOSTRAS

COLONOSCOPIA E BIÓPSIA COLORRECTAL

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

Para doentes com patologia colorrectal a serem submetidos a colonoscopia e eventual biópsia diagnóstica/terapêutica

SERVIÇO DE GASTRENTEROLOGIA – HUC

(Diretor: Prof. Doutor Maximino Correia Leitão)

I – Colheita de amostras em doentes com patologia colorrectal com indicação para colonoscopia e biópsia diagnóstica/terapêutica e que obedecem aos critérios de inclusão:

I.1 – Assinatura prévia do consentimento livre esclarecido, pelo doente participante: o gastroenterologista deverá esclarecer o doente participante sobre o conteúdo do consentimento livre esclarecido; o doente participante deverá ler, preencher e assinar o formulário de consentimento livre esclarecido para validar a sua inclusão no estudo.

I.2 – Colheita de amostra de urina: previamente à realização do exame colonoscópico, o doente deverá receber indicação para a colheita de uma amostra de urina, mediante fornecimento de recipiente apropriado (frasco esterilizado de 60ml); finda a colheita, as amostras serão de imediato entregues ao investigador responsável da FMUC para acondicionamento apropriado.

I.3 – Biópsia sob colonoscopia: durante o exame colonoscópico diagnóstico/terapêutico previsto o gastroenterologista deverá colher:

- 2 biópsias de mucosa normal, quando a patologia for não-tumoral (grupo controlo);
- 2 amostras de lesão, 2 amostras de mucosa colorrectal adjacente à lesão e 2 amostras de mucosa normal à distância, quando a patologia for tumoral ou pré-tumoral (grupo de estudo).

I.4 – Acondicionamento e transporte das amostras biopsadas: finda a colheita, o gastroenterologista deverá acondicionar as amostras de biópsia, sem qualquer tratamento (ex.: imersão em formol), num recipiente de colheita apropriado; de seguida deverá disponibilizá-las ao investigador da FMUC, que será responsável pela sua entrega no Serviço de Anatomia Patológica dos HUC, até 30' após a excisão; na requisição interna, que acompanha as peças ao Serviço de Anatomia Patológica, deverá constar a identificação com as palavras-chave “Projeto Poliomavírus”; o gastroenterologista poderá contactar os investigadores responsáveis, do Serviço de Anatomia Patológica, para avisar sobre o envio das amostras ou para esclarecer qualquer dúvida que surja durante o procedimento.

Investigador Responsável do Serviço de Gastroenterologia - HUC:

Prof. Doutor Pedro Narra de Figueiredo

Telf.: 239400483

E-mail: pnf11@sapo.pt

SERVIÇO DE ANATOMIA PATOLÓGICA – HUC

(Diretora: Dra. Maria Fernanda Xavier da Cunha)

II – Colheita de fragmentos durante exame macroscópico das amostras de biópsia colorrectal:

II.1 – O investigador responsável da FMUC deverá entregar as biópsias, no Serviço de Anatomia Patológica, juntamente com as requisições internas indicando “Projecto Poliomavírus”, as fichas de recolha de dados do doente e os consentimentos livres esclarecidos devidamente assinados.

II.2 - O investigador responsável da FMUC deverá disponibilizar recipientes adequados (tubos eppendorf de 1,5ml) contendo 200µl de solução RNAlater (tampão de estabilização específico para RNA).

II.3 – Nas amostras de biópsia, o anatomopatologista executará a colheita, desde que a mesma não incorra em qualquer prejuízo para o diagnóstico, de 2 fragmentos de mucosa colorrectal correspondentes a lesão, a mucosa adjacente de aspeto colonoscópico normal e a mucosa não-adjacente de aspeto colonoscópico normal.

II.4 – O anatomopatologista deverá colocar cada fragmento, individualmente, num tubo contendo a solução RNAlater.

II.5 - O anatomopatologista atribui um código a cada fragmento, de acordo com a peça de origem, inscrevendo-o em ambos os recipientes, o do fragmento e o da amostra.

II.6 – O investigador responsável da FMUC garante o correto armazenamento e conservação dos fragmentos a -80°C, em caixa adequada e devidamente identificada com o título “Projecto Poliomavírus”.

Investigadores Responsáveis do Serviço de Anatomia Patológica - HUC:

Dr. Mário Rui Silva

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Equipa de Investigação da FMUC:

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Tlm: Prof. Doutor Luís Almeida - 912133241

Tlm: Mestre Tatiana Coelho - 916584854

E-mail: luismeida@ci.uc.pt ou tatiana.coelho@sapo.pt

PROTOCOLO DE COLHEITA DE AMOSTRAS

PEÇA DE RESSECÇÃO CIRÚRGICA

Projeto de Investigação: “Assessment of JC polyomavirus in normal colorectal mucosa, hyperplastic polyps and sporadic adenomas and adenocarcinomas in a Portuguese population and its association with cancer development”

Investigador Principal: Prof. Doutor Luís Manuel da Costa Marques de Almeida, MD, PhD

Doutoranda: Tatiana Andrea Rasteiro Coelho, MSc e Bolseira da Fundação para a Ciência e a Tecnologia

Para doentes a submeter a cirurgia colorrectal de ressecção tumoral

SERVIÇO DE CIRURGIA- CHC

(Diretor: Prof. Doutor Carlos Manuel da Costa-Almeida)

I – Colheita de amostras em doentes submetidos a cirurgia colorrectal de ressecção tumoral e que obedeçam aos critérios de inclusão:

I.1 – Assinatura prévia do consentimento livre esclarecido pelo doente participante: o cirurgião deverá esclarecer o doente participante sobre o conteúdo do consentimento livre esclarecido; o doente participante deverá ler, preencher e assinar o formulário de consentimento livre esclarecido para validar a sua inclusão no estudo.

I.2 – Colheita de amostra de urina: previamente à realização da cirurgia o doente deverá receber indicação para a colheita de uma amostra de urina, mediante fornecimento de recipiente apropriado (frasco esterilizado de 60ml); finda a colheita, as amostras serão de imediato entregues ao investigador responsável da FMUC para acondicionamento apropriado.

I.3 – Colheita de fragmentos a partir da peça operatória: após ressecção da peça operatória o cirurgião selecionará macroscopicamente e fará a colheita de 2 amostras do tumor colorrectal, 2 amostras da mucosa adjacente ao tumor e 2 amostras de mucosa não-adjacente ao tumor, todas elas com aproximadamente 1cm.

I.4 – Acondicionamento das amostras: o cirurgião deverá colocar cada amostra num recipiente apropriado (tubo eppendorf de 1.5ml) contendo 200µl de solução RNAlater (tampão de estabilização

específico para RNA), fornecido pelos Investigadores responsáveis da FMUC; os tubos deverão ser devidamente identificados com o número de registo/entrada, coincidente com o constante na ficha de recolha de dados do doente participante.

I.5 – Recolha das amostras: finda a colheita, o cirurgião deverá contactar os investigadores responsáveis da FMUC, para solicitar a recolha das amostras, devendo entrega-las juntamente com as fichas de recolha de dados e os termos de consentimento livre esclarecido dos doentes; qualquer dúvida que surja durante o procedimento poderá ser esclarecida junto dos investigadores responsáveis da FMUC.

Investigador Responsável do Serviço de Cirurgia do CHC:

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Equipa de Investigação da FMUC:

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CHAPTER 3

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CHAPTER 3

3. Assesment of TP53 mutational status in normal and dysplastic/neoplastic colorectal mucosa

3.1. Introduction

Human colorectal cancer (CRC) has been associated, to some extent, to human neurotropic polyomavirus JC (JCV) infection and transforming potential. The large T-antigen (T-Ag) is its main viral oncoprotein and, like many other oncoproteins, has been recognized as the most crucial for JCV-mediated transformation process, within non-permissive cells, being uniquely able to induce malignant outgrowth and hyperplasia in cultured cells and in several animal models[1,2,3,4,5].

Since JCV is unable to encode its own replication proteins it depends on host-cell machinery to replicate its DNA. Once in host-cells JCV translates its early proteins, namely the T-Ag, a multifunctional protein with helicase and ATPase activity, which are needed for viral DNA replication, and with ability to bind and break DNA[6,7]. Some reports state that T-Ag is not significantly exported from the nucleus into the cytoplasm of the infected human host-cells[8] and that, in fact, it tends to self perpetuate within infected cells and to immortalize the transformed ones, cooperatively with a broad range of cellular proteins, such as p53, pRb, IRS-1 and beta-catenin (Diagram3.1)[9,10,11].

JCV T-Ag can deregulate control of the cell cycle by interacting with the tumour suppressor proteins p53 and pRb, making it unique in the ability to simultaneously disrupt chromosomal integrity and inactivate cell cycle checkpoints. These events can lead either to suppression of p21WAF-1/Cip1, the downstream regulator of p53, or the liberation of the E2F family of transcription factors from pRb. Then, p21WAF-1/Cip1 and E2F stimulate several proteins, essential for cell cycle progression and rapid cell proliferation (Diagram 3.1). Furthermore, these coordinated actions permit the replication and proliferation of cells with damaged DNA[10,12].

T-Ag may also distort control of cellular proliferation by deregulating the Wnt signalling pathway through stabilization of beta-catenin[13], interacting with IGF-IR signalling[14] and inducing chromosomal instability[15]. In fact, it is believed that JCV can mediate colorectal neoplastic process via the deregulation of the Wnt signalling pathway[16]. It has also been proposed that, once colon neoplastic epithelial cells have experienced biallelic inactivation of a critical number of tissue-specific tumour suppressor genes, including APC (Adenomatous Polyposis Coli) and p53, the ongoing effect of the transforming virus may be relatively deleterious[17].

p53 was the first cellular protein identified as interacting with JCV T-Ag and, subsequently, a critical role in deregulation of the cell growth and in genomic instability of infected host-cells was established.

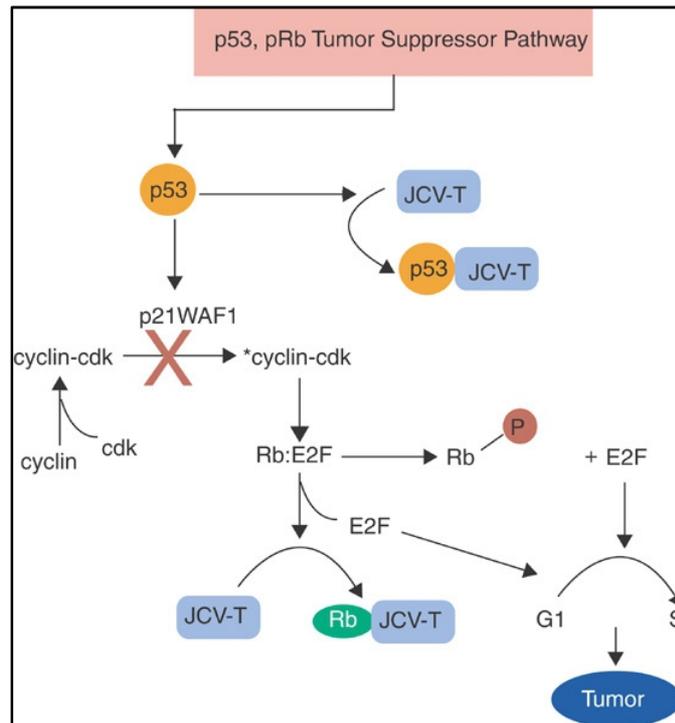


Diagram 3.1 - The p53, pRb tumour suppressor pathways. The association of JCV large T-antigen with p53 leads to inactivation of p53 and downregulation of p21WAF-1 that eventually affects the status of pRb phosphorylation by cyclin cdk and the release of E2F. Further, E2F is liberated from the pRb-E2F complex through the interaction of T-antigen with pRb.

The human p53 protein is coded by a gene located in chromosome 17 (17p13) and that includes 11 exons, with a very large intron between exons 1 and 2 (Diagram 3.2). Exon 1 is non-coding in the human p53 and it has been demonstrated that this region could form a stable stem-loop structure which binds tightly to wild type p53 but not to mutant p53[18].



Diagram 3.2 – Schematized p53 exons: the pink marks denote the UTR's (untranslated regions), the blue marks denote the coding regions and the grey marks denote the internal exons within the introns.

The human wild-type p53 protein contains 393 amino acids and is composed of several structural and functional domains: the amino-terminus part (codons 1-44) contains the transactivation domain, which is responsible for activating downstream target genes; a proline-rich domain (codons 58-101) mediates p53 response to DNA damage through apoptosis; the DNA-binding domain (codons 102-292) is a core domain which consists of a variety of structural motifs (this domain is the target of 90% of p53 mutations found in human cancers, as a single mutation within this domain can cause a major conformational change); and the oligomerization domain (codons 325-356) consists of a β -strand, which interacts with another p53 monomer to form a dimer, followed by an α -helix which mediates the dimerization of two p53 dimers to form a tetramer)[19].

Three putative nuclear localization signals (NLS) have been identified in the C-terminus, through sequence similarity and mutagenesis. The most N-terminal NLS (NLSI), which consists of three consecutive lysine residues to a basic core, is the most active and conserved domain. Furthermore, two putative nuclear export signals (NES) have been identified. The leucine-rich C-terminal NES, found within the oligomerization domain, is highly conserved and it has been suggested that oligomerization can result in masking of the NES, resulting in p53 nuclear retention (Diagram 3.3)[18,19].



Diagram 3.3 – Schematic representation of the p53 structure. p53 contains 393 amino acids, consisting of three functional domains: an N-terminal activation domain, DNA binding domain and C-terminal tetramerization domain. The N-terminal domain includes transactivation sub-domain and a PXXP region that is a proline-rich fragment. The central DNA binding domain is required for sequence-specific DNA binding and amino acid residues within this domain are frequently mutated in human cancer cells and tumour tissues. The Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282 are reported to be mutation hot spots in various human cancers. The C-terminal region is considered to perform a regulatory function. Residues on this basic C-terminal domain undergo posttranslational modifications including phosphorylation and acetylation.

Previous studies showed that human wild-type p53 is able to repress JCV DNA replication *in vivo* by interacting with JC virus T-Ag which is necessary for initiation and elongation of viral DNA replication. Using deletion mutants and tumour-derived point mutations of human p53, the basis of the suppression of JCV DNA replication by p53 is suppressed. Deletion of either the amino- or the carboxyl-terminal domain of p53 does not interfere with the repression of JCV DNA replication. However, deletion of the highly conserved central region of p53 abolished the inhibitory effect on replication. The tumour-derived human mutant p53(His273) inhibited JCV DNA replication significantly, whereas the tumorigenic mutant p53(His175) has no inhibitory effect. Concomitantly, a direct protein–protein interaction between p53 and JCV large T-antigen is lost when using mutants which did not affect JCV DNA replication. These results strongly suggest that p53 is able to inhibit JCV DNA replication by interacting with JCV T-Ag[20].

However, the inhibition of p53 function by T-Ag is necessary for both efficient viral replication and cellular transformation of host-cell infected by JC polyomavirus. The analysis of the crystal structure of T-Ag in complex with p53 reveals an unexpected hexameric complex of T-Ag binding six p53 monomers. The structure also shows that T-Ag binding induces dramatic conformational changes at the DNA-binding area of p53, which is achieved partially through an unusual “methionine switch” within p53. In the complex structure, T-Ag occupies the whole p53 DNA-binding surface and likely interferes with formation of a functional p53 tetramer[21].

3.2. Purpose

It seems that the different outcomes of the JC viral infection probably depend on multiple factors, both pathogen-related and host-related. Genomic and proteomic biomarkers are proving to be particularly useful in the understanding of disease progression, staging and response to therapy in chronic diseases and cancer.

Since disease/infection progress depends on the host response to viral infection, the assesment of the genetic status of JCV infected cells in asymptomatic individuals is a promising starting point to look for potential biomarkers for viral infection.

The main purpose of this experimental work was to evaluate the host-cell genetic conditions that may contribute for the progression from a latent to an abortive/transforming JCV infection and act as risk modifiers in colorectal carcinogenesis, mediated by JC virus in the infected individuals. Thus, we screened for genetic alterations (mutations/polymorphisms) in TP53 that can be potentially able to modify p53 protein affinity for and interaction with the viral oncoprotein T-Ag.

To our knowledge the assesment of host-cell genetic features that can act as potential risk modifiers for CRC development in JCV infected individuals, the identifications and establishment of new risk factors for JCV-mediated colorectal carcinogenesis in humans may contribute to the a short-term improvement of both early and differential diagnostic, prognostic and clinical surveillance.

Once JC virus can participate in different ways in the pathogenesis of colorectal cancer, both direct and indirect we additionally assessed the mutational profile of the TP53 gene in the normal colorectal mucosa, adenomas and adenocarcinomas from infected and non-infected individuals, both from the control and the study group, in order to evaluate the extension of the contribution of the JCV infection to the mutagenesis during colorectal cancer development.

3.3. Samples and Methods

3.3.1. DNA samples

The mutational screening of the TP53 gene was performed by PCR amplification, followed by DNA Sequencing, using total genomic DNA obtained, as previously described in Chapter 2, from: a) the normal colorectal mucosa of 100 controls (40 of them positive for JCV DNA) and 100 patients from the study group (41 of them positive for JCV DNA); and b) dysplastic/neoplastic colorectal lesions consisting in 60 adenomas (55 of them positive for JCV DNA) and 14 adenocarcinomas (12 of them positive for JCV DNA) from patients of the study group (Chapter 2).

3.3.2. Mutational Analysis of the p53 gene

3.3.2.1. PCR Amplification of TP53 exons

In order to amplify TP53 exons, we designed several pairs of TP53-specific exon primers (forward and reverse), targeted to exons 1 to 11 (prepared by StabVida, Caparica, Portugal) (Table 3.1). PCR primers anneal to the intron sequences, immediately upstream and downstream of each exon, and enclosed fragments with sizes ranging from 186-500b.p. As most inactivating genetic alterations occur within the DNA binding domain (codons 102-292), and considering that this is the interaction domain with JCV T-Ag, we focused our attention to the exons encoding this region of the protein, which comprises exons 5 through 8.

The PCR reaction, for the amplification of each TP53 exon, was carried out individually in a PCR mixture of 50µl of final volume containing 1× PCR incomplete buffer (50mM KCl/10mM Tris HCl/2mM) (Bioron, Ludwigshafen, Germany), 2mM of Mg²⁺ (Bioron, Ludwigshafen, Germany), 200µM of dNTPs (Bioron, Ludwigshafen, Germany), 20pmol of each primer (forward and reverse), 2.5units of Taq DNA polymerase (Bioron, Ludwigshafen, Germany), 50ng of DNA obtained from each normal colorectal mucosa sample and Mili-Q water until complete the final volume. The reactions were then submitted to thermal cycling: a Hot-Start (96°C for 1'), followed by 40 cycles of denaturing at 96°C for 1', annealing at 60°C for 30'' and extension at 72°C for 1', with a final extension step at 72°C for 10'.

All PCR's included a "no template" control (with no target DNA added), to ensure that our solutions were not contaminated with other DNA that might serve as a template.

The success of the PCR amplification was confirmed by horizontal electrophoresis on agarose gels, as previously described in Chapter 2. TP53 amplimers were identified by comparison with the fragments of a known molecular weight marker, 100b.p. ladder (Bioron, Ludwigshafen, Germany). Finally, all the PCR products were purified with ExoSAP[®]-IT For PCR Product Clean-Up Kit, according to manufacturer's instructions, aiming to remove single-stranded DNA (i.e., primers and incomplete PCR products) and unconsumed dNTPs.

3.3.2.2. TP53 Sequencing

Before sequencing, the templates were treated with BigDye[®] Terminator Ready Reaction Mix v3.1 (Applied Biosystems, Germany), according to manufacturer's protocol for short templates, as those obtained from the PCR amplification using our TP53- specific exon primers (<500b.p.). Then, each sequencing PCR reaction was prepared individually, for a final volume of 20µl, and contained 5x Buffer (Applied Biosystems, Germany), 2.5ng of the primer (forward or reverse), 4µl of the treated template and Mili-Q water to complete the final volume. The reactions were then submitted to 25 thermal cycles of 96°C for 10'', 50°C for 5'' and 60°C for 4'. These sequencing PCR products were cleaned-up with Sefadex[®] resin and centrifuged at 2000rpm (1539g) for 1'.

Finally, all templates were sequenced in an Applied Biosystems® 3130 Genetic Analyser (Applied Biosystems, Germany), in both directions (with forward and reverse primers) to avoid ambiguities.

3.4. Results

3.4.1. TP53 genetic alterations in normal colorectal mucosa from infected and non-infected individuals

In a total of 81 normal colorectal mucosa specimens infected by JC virus, from 40 controls and 41 patients, the mutational analysis of the TP53 gene by DNA Direct Sequencing allowed the detection of several genetic alterations in 10 of the 40 controls (25%) and 30 of the 41 patients (73%). Furthermore, from the 119 normal colorectal mucosa samples with no positivity for JCV DNA (60 controls and 59 patients) we were able to identify a single TP53 defect in 5 of the 60 controls (8%) and 13 of the 59 patients (22%).

Our study revealed several variants of the TP53 gene: a) three intronic single nucleotide polymorphisms (SNPs) in intron 2 – an homozygous C insertion at position 11818 (Figure 3.1A), an G>C substitution at position 11827 (Figure 3.1B) and an homozygous C insertion at position 11875 (Figure 3.1C); b) a synonymous A>G substitution at position 13399 in the exon 6 (Figure 3.1D); and c) a new homozygous C insertion at position 19037 in the 3'UTR (3' untranslated region) of the exon 11 (not described to date), exclusively found in the normal colorectal mucosa of 10 JCV infected patients (Figure 3.1E). The C insertion at position 11875 in intron 2 was the only variant identified in individuals with no positivity for JC virus. No other genetic alterations were detected in TP53 gene, even within the DNA binding domain, which comprises exons 5 through 8 and where interaction with JCV T-Ag occurs.

The absolute frequencies of these TP53 variants, identified in the normal colorectal mucosa of JCV infected and non-infected individuals, are presented in Table 3.2A, as well as the allelic and genotypic frequencies in Table 3.2B.

3.4.2. TP53 mutational status in adenomas and adenocarcinomas from JCV infected *versus* non-infected patients

From a total of 74 dysplastic/neoplastic colorectal lesions, 60 adenomas (55 JCV+ and 5 JCV-) and 14 adenocarcinomas (12 JCV+ and 2 JCV-) from patients included in the study group, the mutational analysis of the TP53 gene allowed the detection of genetic alterations in all the studied samples.

We were able to identify four concomitant TP53 genetic alterations: a) the G>C substitution at position 11827 in the intron 2(Figure 3.2A); b) a C>G substitution at position 12139 in the exon 4 (Figure 3.2B); c) the synonymous A>G substitution at position 13399 in the exon 6 (Figure 3.2C); and d) the new homozygous C insertion at position 19037 in the 3'UTR of the exon 11 (Figure 3.2D). Again, the TP53 3'UTR variant was exclusively detected in colorectal lesions from 62 patients with positivity for the presence of JC virus DNA. No other genetic alterations were detected in TP53 gene.

The absolute frequencies of TP53 variants detected in the dysplastic/neoplastic colorectal mucosa of the JCV infected and non-infected patients from the study group are listed in Table 3.3A and the allelic and genotypic frequencies in Table 3.3B.

3.5. Discussion

Concerning to TP53 mutational analysis reports state that more than 90% of the mutations reported in p53 gene are clustered between exons 4 and 8. This region is highly conserved throughout evolution and contains the DNA-binding domain of p53, which is essential for its activity. This contrasts with the trans-activation domain (encoded by exons 2 and 3) and the regulatory region (encoded by exons 9 to 11), where few mutations have been described[22].

The new TP53 single nucleotide polymorphisms identified in our experimental approach, a homozygous C insertion at position 1903 in the 3' UTR of exon 11 (19037insC Ex11), revealed to be a significantly recurrent and exclusive characteristic of the colorectal mucosa, either normal or dysplastic/neoplastic, of the JCV infected individuals with clinical history of colorectal lesions, when comparing with controls and non-infected patients.

Bibliographic reports state that the *cis*-regulatory elements in the 3'-UTRs play an important role in the translation, localization and stability of the mRNA and genetic alterations within this region can be the cause of several diseases, such as congenital heart disease (CHD), associated with 3'-UTR variations in GATA4 gene[23], and arrhythmogenic right ventricular cardiomyopathy/dysplasia (AVRC), also invariably related with abnormal TGF-beta3 3'-UTR[24].

The research on the pathophysiology of diseases and mutations affecting the functionality of the 3'-UTR is still sparse. However, the available data do suggest that this mRNA region, which is often neglected during the genetic screening of disease-associated candidate genes, plays an important role in various diseases and disease progression[25,26].

The 3'-UTR is characterized by secondary structures which play an important role in the interaction of mRNAs with the associated proteins. A disturbance in these structures, due to inherent genetic changes in sequence, alters its interaction with other proteins. A study by Chen *et al*[27] on eighty three disease-associated variants in the 3'-UTR of various human mRNAs revealed a

correlation between the functionality of these variants and alterations in the predicted secondary structure. Such studies emphasize the importance of this region in translational regulation.

Using a prediction bioinformatic tool (*3D-JIGSAW* Protein Comparative Modelling Server – Cancer Research UK) for a secondary structure preview of the TP53 3'-UTR, both for wild-type and mutant 19037insC sequences, we found significant differences between the predictive secondary structures, probably due to the considerable changes in codon frame. Since mutations affecting the secondary structure of 3'-UTR of mRNA can cause translation deregulation and diseases, we may presume that the 19037insC in the 3' UTR of TP53 gene may be related in some extent with a predisposition to a p53/JCV T-Ag-mediated colorectal carcinogenesis, and act as a risk modifier in JCV infected individuals. In fact, all the available information suggest a direct association between the mutations in 3'-UTR and disease development. However, this possibility needs further confirmation.

Although located in a non-coding region, one might consider that the three intronic single nucleotide polymorphisms found in TP53 intron 2 can become noticed in p53 protein function. Perhaps changes in the nucleotide sequence of the genome of p53 can altered the splicing signals required for appropriate splicing of p53 and caused more severe genetic alterations. In fact, the C insertions found at positions 11818 and 11875 of TP53 intron 2 (11818insC Int2 and 11875insC Int2), were previously described by Soto *et al*[22] as involved in melanoma tumourigenesis. The G to C transversion found at position 11827 in TP53 intron 2 (11827G>C Int2), at b.p. +38 following the splice donor site of exon 2, was also already described by Pleasants *et al*[28] and Oliva *et al*[29] as a polymorphic site useful as a marker to detect the loss of heterozygosity in human tumours, such as soft tissue sarcomas, bladder carcinomas and colorectal carcinoma. Despite the 11827G> C Int2 variant has been detected in the normal mucosa of individuals from the control group, positive for the presence of JC virus DNA, its relative frequency is not representative.

Concerning to the synonymous A>G substitution at position 13399 in the exon 6 (13399A>G Ex6), a silent variant at codon 213 (CGA to CGG) that codes the aminoacid arginine, although located within p53 interaction domain with JCV T-antigen, is clear that nucleotide change does not alter the aminoacid transcript and, consequently, no changes shall occur in the p53 structure and affinity to JCV T-Ag [30].

The C>G substitution at position 12139 in the exon 4 (12139C>G Ex4) is described as a missense genetic alteration at codon 72 (CCC to CGC) which promotes the change from the aminoacid proline (variant p53-P72) to arginine (variant p53-R72). The p53-P72 variant has been reported as the ancestral form, although the p53-R72 may occur at a high frequency (>50%) in some populations[31,32].

The codon 72 in TP53 exon 4 is located within the gene segment that encodes the polyproline domain, which lies between the N-terminal transactivating domain and the DNA-binding domain, in which most tumour-associated mutations are found. The precise contribution of the polyproline domain to p53 regulation and function is still unclear. Unlike the DNA-binding domain, the polyproline domain is less well-conserved across species than the DNA-binding domain and is not a common location of tumour-associated mutations. Deletion studies in cells and mice support the view that the polyproline domain is essential for p53 to mount a full apoptotic response to stress and inhibit tumourigenesis[33,34,35,36].

A decade ago, a dramatic effect of the p53 codon 72 variant on the risk of cervical cancer was reported. This effect was explained by the finding that the E6 oncoprotein from high-risk mucosal Human Papillomaviruses (HPVs) causes more efficient degradation of p53-R72 than p53-P72, reducing cellular levels of p53 and increasing the risk of HPV-associated cancers in p53-R72 homozygotes[37]. These findings triggered an intensive investigation on the potential effect of the p53 codon 72 polymorphism on susceptibility to various cancers or cancer-related phenotypes that is still ongoing. Interestingly, our results suggest that the codon 72 variant of TP53 gene may be associated with sporadic colorectal adenoma/adenocarcinoma risk in carriers of the Arginine/Arginine genotype. In fact, it remains plausible that the codon 72 variant is a modifier of cancer risk or age of onset when considered in the context of other genetic and cancer-associated mutations in other genes [38,39,40].

Recently, Vilkin and colleagues[41] tried to clarify the role of JCV in tumour development in patients with sporadic colorectal cancer and with positive family history and Bethesda criteria. They were unable to conclude that, on a background of genetic mutations in mismatch repair genes, JCV acts as an additional event leading to CRC, but yet CRC patients with positive family history have a higher incidence of JCV T-Ag. This suggests a potential collaboration between some predisposition genes and JCV. Our finding of some p53 intronic nucleotide changes detected more frequently in colorectal lesions, but also present in controls, is consistent with this interpretation. In colorectal cancer p53 mutations are frequent at late stages of tumour development.

Thus, our finding that no p53 mutations were detected in mostly early lesions is consistent with this late role. However, it is interesting to note that most p53 nucleotide alterations were identified within intron 2, and were also present in the normal population. These are likely polymorphisms that indirectly might facilitate, or contribute to, transformation of JCV-infected normal cells. A similar role might be played by the new and common mutation identified only in adenoma or carcinoma cases that is located within the 3' un-translated region. One common mechanistic aspect to all these different nucleotide changes in non-coding regions is that they might affect RNA stability, leading to a lower level of p53 protein and somehow making p53-mediated responses less efficient, and in that way predisposing to tumour initiation or progression.

3.6. Conclusions

It is generally accepted that the inactivation of the tumour suppressor gene TP53, caused by genetic variations, is a common event in the genesis of several human tumours. As previously reported in several studies, the occurrence of intronic genetic alterations in the p53 genome may result in an alternative pattern of splicing leading to the generation of a p53 variant which encompasses the T-antigen binding site. It is proved that variations in the close proximity of or within the T-antigen binding site may affect proper folding of the p53 protein and its binding to JCV T-antigen.

Our experimental approach revealed that the most relevant genetic alterations found in TP53 gene, such as the new SNP identified in TP53 3' UTR (19037insC Ex11) and the TP53 codon 72 variant (12139C>G Ex4), seem to be a common and exclusive characteristic of the colorectal epithelium cells of JCV infected individuals, especially those with clinical history of colorectal lesions. The TP53-3' UTR variant, with its contribution to changes in the secondary structure of the 3'-UTR, may be associated, in some extent, with a p53/JCV T-Ag-mediated colorectal carcinogenesis in JCV infected individuals. Additionally, our results suggest that the codon 72 variant of TP53 gene may be associated with a sporadic colorectal adenoma/adenocarcinoma risk in carriers of the Arginine/Arginine genotype. In fact, it is believed that the codon 72 variant is a modifier of cancer risk or age of onset when considered in the context of other genetic and cancer-associated mutations in other genes.

We may suggest that these p53 variants may, probably, participate as risk modifiers in the JCV-mediated colorectal cancer development, but still, the implication of such variants in this context requires further elucidation.

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3.8. Web Site Support

- The National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>.
- The p53 Web Site: <http://p53.free.fr/>.
- The Human Gene Mutation Database (HGMD): <http://www.hgmd.cf.ac.uk/ac/index.php>.
- in-silico Project Support for Life Sciences: http://in-silico.net/tools/biology/sequence_conversion.
- 3D-JIGSAW Protein Comparative Modelling Server: <http://bmm.cancerresearchuk.org/~3djigsaw/>.

3.9. Figures and Tables

TABLE 3.1. Oligonucleotide sequences for mutational screening of TP53 gene by PCR-DNA sequencing.

Gene	Target DNA Sequence	Primer Name	Primer Sequence
TP53	Exon 1 (186b.p.)	P53E1F	5'-gctcaagactggcgctaaaa-3'
		P53E1R	5'-gtgactcagagaggactcat-3'
	Exons 2 and 3 (352b.p.)	P53E2/3F	5'-aagtgtctcatgctggatcc-3'
		P53E2/3R	5'-aaagagcagtcagaggacca-3'
	Exon 4 (351b.p.)	P53E4F	5'-tggtcctctgactgctcttt-3'
		P53E4R	5'-tgaagtctcatggaagccag-3'
	Exons 5 and 6 (487b.p.)	P53E5/6F	5'-cacttgctgccctgacttca-3'
		P53E5/6R	5'-ccactgacaaccacccttaa-3'
	Exon 7 (351b.p.)	P53E7F	5'-cttgactgagctgagatca-3'
		P53E7R	5'-agaaatcggttaagaggtggg-3'
	Exons 8 and 9 (417b.p.)	P53E8/9F	5'-taaatggacaggtaggacc-3'
		P53E8/9R	5'-tccactgataagaggtccc-3'
	Exon 10 (232b.p.)	P53E10F	5'-gagtgagacccatctcaaa-3'
		P53E10R	5'-ttccaacctaggaagcag-3'
	Exon 11 (500/471/499b.p.)	P53E11F1	5'-gaccctctactcatgtgat-3'
		P53E11R1	5'-gcatttcacagatatgggcc-3'
		P53E11F2	5'-gaagctgtccctcatgttga-3'
		P53E11R2	5'-aaagcgagaccagctctcaa-3'
P53E11F3		5'-ctggatccaccaagactgt-3'	
P53E11R3		5'-tccacactcattgcagactc-3'	

b.p.=Base Pairs

F=Forward

R=Reverse

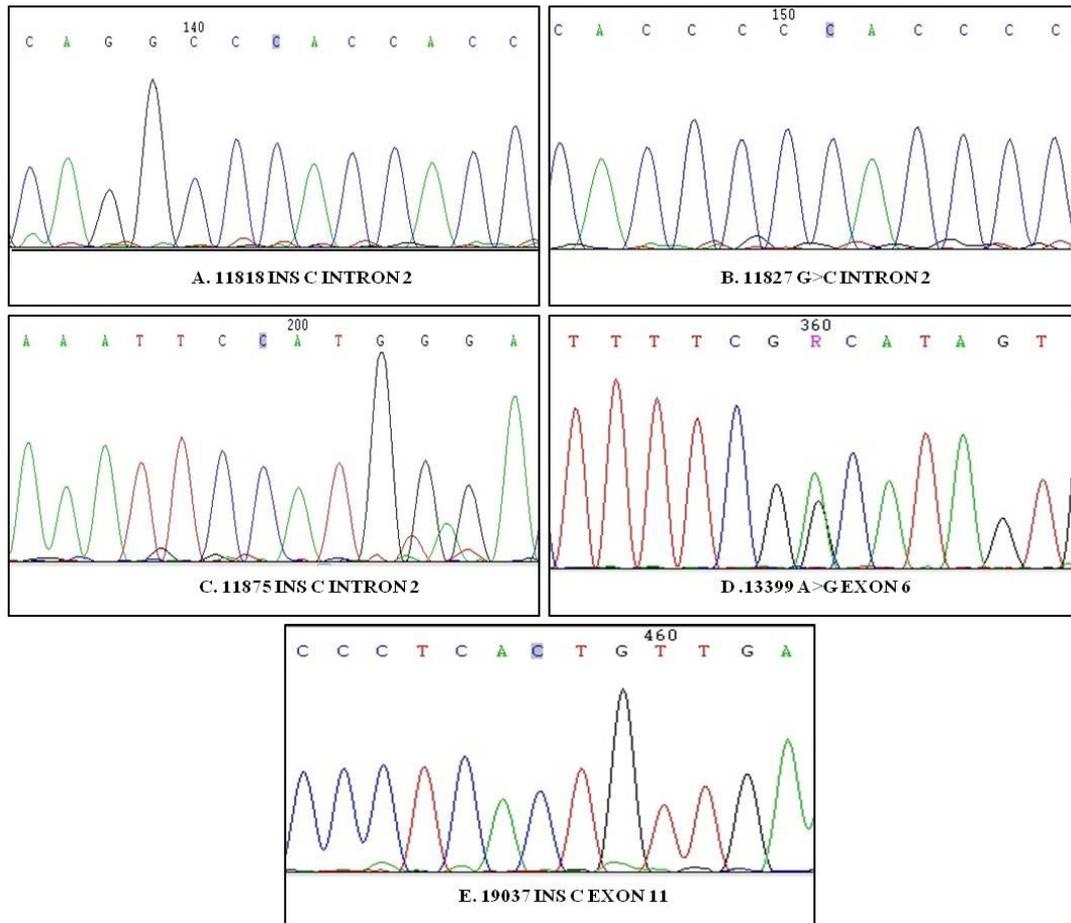


Figure 3.1

Diagrams of DNA sequencing electrophoresis showing TP53 genetic variants found in the normal colorectal mucosa from patients and controls infected and non-infected by JC virus.

A=Adenine; T=Thymine; C=Cytosine; G=Guanine; R=A/G

TABLE 3.2A. Absolute frequencies of TP53 variants in the normal colorectal mucosa of JCV infected and non-infected individuals from both control and study groups.

TP53 Genetic Variants	Absolute Frequencies			
	Control Group (n=100)		Study Group (n=100)	
	JCV+ (n=40)	JCV- (n=60)	JCV+ (n=41)	JCV- (n=59)
11818insC Int2	1	0	3	0
11827G>C Int2	3	0	5	0
11875insC Int2	1	5	1	13
13399A>G Ex6	5	0	11	0
19037insC Ex11 (new)	0	0	10	0
TOTAL	n=10	n=5	n=30	n=13

TABLE 3.2B. Allelic and genotypic frequencies of TP53 variants in the normal colorectal mucosa of JCV infected individuals from both control and study groups.

TP53 Genetic Variants	Control Group JCV+ (n=40)		Study Group JCV+ (n=41)	
	Allelic*	Genotypic**	Allelic*	Genotypic**
11827G>C Int2	G – 0.96	GG – 0.925	G – 0.89	GG – 0.878
	C – 0.04	GC – 0.075	C – 0.11	GC – 0.024
		CC – 0		CC – 0.098
	n=3		n=5	
13399A>G Ex6	A – 0.94	AA – 0.875	A – 0.76	AA – 0.731
	G – 0.06	AG – 0.125	G – 0.24	AG – 0.048
		GG – 0		GG – 0.221
	n=5		n=11	

JCV+=Positive for JCV DNA

JCV-=Negative for JCV DNA

***Allelic Frequency:**
 $f(B) = ((2 \times \text{number of homozygous BB}) + (\text{number of heterozygous Bb})) / (2 \times \text{total of individuals})$
 $f(b) = ((2 \times \text{number of homozygous bb}) + (\text{number of heterozygous Bb})) / (2 \times \text{total of individuals})$
 $f(B) + f(b) = 1$
****Genotypic Frequency:**
 $f(BB) = \text{number of homozygous BB} / \text{total of individuals}$
 $f(Bb) = \text{number of heterozygous Bb} / \text{total of individuals}$
 $f(bb) = \text{number of homozygous bb} / \text{total of individuals}$
 $f(BB) + f(Bb) + f(bb) = 1$

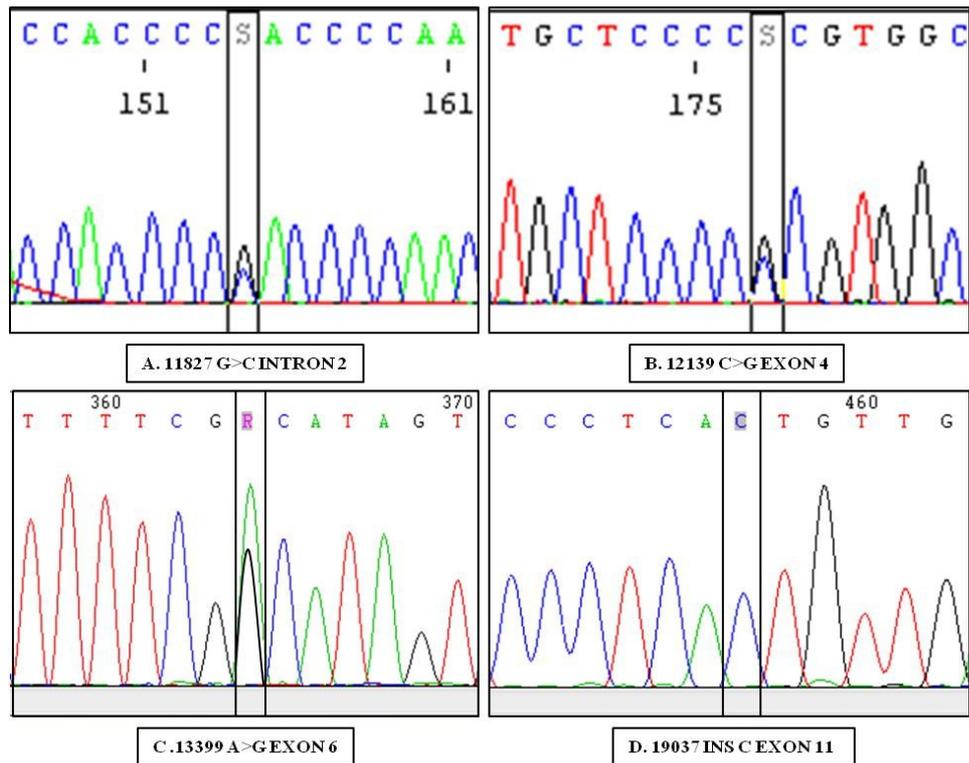


Figure 3.2

Diagrams of DNA sequencing electrophoresis showing the TP53 genetic alterations found in colorectal adenomas and adenocarcinomas from JCV infected and non-infected patients.

A=Adenine; T=Thymine; C=Cytosine; G=Guanine; S=G/C; R=A/G

TABLE 3.3A. Absolute frequencies of TP53 variants in adenomas and adenocarcinomas from JCV infected and non-infected patients from the study group.

TP53 Genetic Variants	Absolute Frequencies			
	Adenomas (n=60)		Adenocarcinomas (n=14)	
	JCV+ (n=55)	JCV- (n=5)	JCV+ (n=12)	JCV- (n=2)
11827G>C Int2	21	0	10	0
12139C>G Ex4	38	0	12	0
13399A>G Ex6	49	5	11	2
19037insC Ex11 (new)	55	0	12	0
TOTAL	n=55	n=5	n=12	n=2

TABLE 3.3B. Allelic and genotypic frequencies of TP53 variants in adenomas and adenocarcinomas of JCV infected patients from the study group.

TP53 Genetic Variants	Adenomas (n=60)				Adenocarcinomas (n=14)			
	Allelic*		Genotypic**		Allelic*		Genotypic**	
	JCV+ (n=55)		JCV+ (n=55)		JCV+ (n=12)		JCV+ (n=12)	
11827G>C Int2	G-0.69		GG-0.618		G-0.21		GG-0.166	
	C-0.31		GC-0.146		C-0.79		GC-0.084	
			CC-0.236				CC-0.750	
			n=21				n=10	
12139C>G Ex4	C-0.49		CC-0.309		C-0.04		CC-0	
	G-0.51		CG-0.364		G-0.96		CG-0.083	
			GG-0.327				GG-0.917	
			n=38				n=12	
13399A>G Ex6	Allelic*		Genotypic**		Allelic*		Genotypic**	
	JCV+ (n=55)	JCV- (n=5)	JCV+ (n=55)	JCV- (n=5)	JCV+ (n=12)	JCV- (n=2)	JCV+ (n=12)	JCV- (n=2)
	A-0.21	A-0.20	AA-0.109	AA-0	A-0.13	A-0.50	AA-0.083	AA-0
	G-0.79	G-0.80	AG-0.200	AG-0.400	G-0.87	G-0.50	AG-0.083	AG-1
			GG-0.691	GG-0.600			GG-0.834	GG-0
	n=49	n=5	n=49	n=5	n=11	n=2	n=11	n=2

JCV+=Positive for JCV DNA

JCV-=Negative for JCV DNA

***Allelic Frequency:**

$$f(B) = ((2 \times \text{number of homozygous BB}) + (\text{number of heterozygous Bb})) / (2 \times \text{total of individuals})$$

$$f(b) = ((2 \times \text{number of homozygous bb}) + (\text{number of heterozygous Bb})) / (2 \times \text{total of individuals})$$

$$f(B) + f(b) = 1$$

****Genotypic Frequency:**

$$f(BB) = \text{number of homozygous BB} / \text{total of individuals}$$

$$f(Bb) = \text{number of heterozygous Bb} / \text{total of individuals}$$

$$f(bb) = \text{number of homozygous bb} / \text{total of individuals}$$

$$f(BB) + f(Bb) + f(bb) = 1$$

CHAPTER 4

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CHAPTER 4

4. Final Considerations

4.1. Overall Findings

JC polyomavirus (JCV) is a significant human pathogen with an intricate biology. However, a causative role for JCV in human cancers has not yet been established. What criteria are necessary to establish causality or an association of JCV with human cancers? Some of the necessary criteria to establish a causal relationship between a virus and cancer include the detection of viral genome or gene products in cancer tissue, a molecular basis for virus-induced oncogenicity and consistency of the association.

Does JCV fit these criteria? In addition to the evidence that JCV can cause tumours in experimental animals and transform cells in culture, the viral genomic DNA and the expression of the viral proteins has been demonstrated in several tumour tissues. The oncogenic properties of JCV are well described in the literature and attributed to its oncoprotein, the large T-antigen (T-Ag), which directly interacts with tumour suppressor proteins and cell-cycle regulators to disrupt the cell cycle and prevent apoptosis. Laboratory studies of this viral protein have demonstrated that T-Ag has the ability to immortalize cells in culture and its expression in transgenic mice results in the development of tissue-specific tumours. However, a reliable association of JCV with a particular type of tumour or human cancer has not been consistently established. In fact, JCV has been reported to contribute to a wide variety of tumours indicating that there is not a clear correlation of JCV with a particular type of cancer.

The design of the present study was based in the fact that there are relatively few reports that implicate JCV in human cancers, a comparable number of studies that refute these findings and a lack of epidemiological studies that, specifically, link JCV to human colorectal cancer (CRC). Still, we found reasonable to hypothesize that JCV may act as a co-factor in human colorectal tumourigenesis.

The results of our study allowed us to consider that the presence of JCV DNA in colorectal lesions is an early and sustained event during tumour progression, unrelated with the lesion type, grade or severity, and that JCV may have a specific tropism for CRC and colorectal polyps, either hyperplastic or dysplastic (adenomas). This observation may reflect a condition where the transformed colorectal epithelial cells offer a more permissive environment for viral replication, being effective even since the hyperplastic polyps not prone to carcinogenesis. In fact, all together, our findings suggest that JC polyomavirus infection may precede the colorectal lesion occurrence, probably acting as a risk co-factor, or just be related with its progression, acting as a carcinogenic enhancer.

Furthermore, we propose the epidemiological extent of the JCV infection in the gastrointestinal and urinary tracts of the normal Portuguese population, and the occurrence of a biological selection of JCV infected cells during tumourigenesis in individuals with colorectal lesions.

Additionally, our experimental approach revealed that the most relevant genetic alterations found in TP53 gene, such as the new single nucleotide polymorphism identified in TP53 3'-untranslated region (19037insC Ex11) and the TP53 codon 72 variant (12139C>G Ex4), seems to be a common and exclusive characteristic of the colorectal epithelium cells of JCV infected individuals, especially those with clinical history of colorectal lesions. These p53 variants can participate, in some extent, as risk modifiers in JCV-mediated colorectal cancer development. Still, the implication of such variants in the context of the JCV-mediated colorectal carcinogenesis requires further elucidation.

On the other hand, finding that no p53 mutations were detected in mostly early lesions is consistent with the late role of the p53 protein in colorectal carcinogenesis, which, at the same time, suggests an early role for JCV contribution to this type of tumour. However, it is interesting to note that most p53 nucleotide alterations are identified within intron 2 and are also present in JCV-infected controls. These polymorphisms are likely to indirectly facilitate, or contribute to, the transformation of JCV-infected normal cells. A similar role might be played by the new and common mutation, located within the 3'-untranslated region and identified only in adenomas and adenocarcinoma of JCV infected patients.

One common mechanistic aspect to all of these different nucleotide changes in non-coding regions is that they might affect RNA stability or result in RNA-mediated decay. The net effect would probably be a reduced level of cellular p53 protein, making, somehow, p53-mediated responses less efficient and predisposing to tumour initiation or progression.

These associations may be too speculative considering that no other evidences has been reported or studied yet. In fact, we do not know if these p53 variants, present in colorectal epithelium cells, are restricted to the Portuguese or Iberian populations or if its presence is more widespread, being possible to detect it in other populations. If so, it has not been reported or given any attention by itself or in the context of colorectal cancer.

4.2. General Considerations

The recent identification of many components of the *Polyomaviridae* family that are able to infect humans represents a strong incentive for the scientific community to improve and increase the research on the oncogenic potentialities of these viruses. The ubiquity and persistent nature of polyomaviruses make them very challenging in order to define the mechanisms of their pathogenicity. In particular, since the association between the viral aetiology of the tumours and the state of the host immune system is well established, it is urgent to focus future studies on the nature of the relationship between the host immune system and polyomaviruses infection.

JCV reactivation in non-permissive cells after treatment with immuno-modulatory therapies, such as colorectal epithelial cells in Crohn's disease patients, in association with other host tumour-inducing factors, could provide valid information on the role of JCV in several malignancies, such as colorectal cancer.

Studies analyzing the association of JCV with human tumours in various states of immuno-competency would be worthwhile and may reveal interesting new insights into the role of JCV in human cancers. On the other hand, the inflammatory infiltration caused by the local JCV chronic infection of the colorectal mucosa can contribute, itself, to the selection and expansion of tumour-prone cells. This is an issue that has not been explored in the context of gastrointestinal JCV infection and which deserves further attention, particularly if considering that chronic inflammation is a risk factor in colorectal cancer.

The inactivation of the tumour suppressor gene TP53 caused by genetic variations is a common event in the genesis of several human tumours. As previously reported in several studies, the occurrence of intronic genetic alterations in the p53 genome may result in an alternative pattern of splicing, leading to the formation of p53 variants which encompasses the T-Ag binding site. It is proved that variations in the close proximity of or within the T-Ag binding site may affect proper folding of the p53 protein and its binding to JCV T-Ag.

To our knowledge, it is appropriate to consider that JCV can initially infect uncontrolled dividing tumour cells and then the expression of viral early genes may contribute to oncogenesis. It is known that CRC originates in epithelial cells which replicates at a relatively high rate, with 10^{10} epithelial cells being replaced every day. This high rate of replication is thought to contribute to the vulnerability of colon epithelium to mutation and consequent carcinogenesis. If colonic epithelial cells accumulate mutations in oncogenes and tumour suppressor genes, the morphology of the cell changes, and there is a hyper-proliferation of the abnormal ones. If JCV infection occurs in a cell that is already undergoing aberrant cell growth, due to misregulation of the cell cycle or other genetic or

environmental predisposition factors, JCV may provide the “second hit” that prompt to cancer development.

Therefore, it is worthwhile to carry-out gene expression assays in order to demonstrate the differences between normal and tumoural colorectal tissue or to clarify the interaction between p53 protein and JC polyomavirus. It is doubtless crucial to continue exploring the role of JCV in human cancers as a co-factor in oncogenesis, especially since ever-changing environmental and social factors play such an important role in disease pathogenesis and cancer.

In order to properly establish if JCV is indeed an important risk co-factor in colorectal cancer there are several issues that need to be properly addressed: a) establish conclusively that CRC patients, in a significant number of cases, have been exposed to this virus, and that an indirect marker will be the presence of specific antibodies; b) improve detection of viral DNA, by using standardized sets of primers for each of the relevant viral genes, as well as strain identification; this should also contribute to establish the potential and irreversible integration of remnant JCV DNA in cancer cells, and permit identification of significant differences among malignant, benign or normal surrounding tissue; c) correlate JCV DNA presence with other mutations known to sequentially occur in CRC, and its association with other known risk factors; d) identify the viral protein expression at different stages of colorectal cancer progression; for this purpose, the development of more specific antibodies for JCV proteins are necessary; and e) characterize the host immune response to JCV in order to manipulate it and develop strategies to eradicate the virus from the human population, particularly to determine the role T-cell responses, since the natural antibody response does not seem to be protective.

We consider that future studies need to establish a standard, reliable, and reproducible test for the detection of JC virus DNA. This test should be evaluated in masked specimens, multiple populations and different laboratories to insure the validity of the results. In addition, prospective studies of faecal carriage of JC virus in relation to colorectal cancer are absent from the literature. Such studies could help to determine the specificity, and possibly the causality, of the association between JC virus and colorectal cancer, and if faecal carriage of virulent JCV subtypes occurs prior to the development of colorectal neoplasia and at higher rates in cases than in controls.

Finally, we consider that the short-term planning of strategies to reduce the burden of JCV in the population might contribute to reduce the incidence of colorectal cancer, similarly to other human cancer associated to infectious agents. In addition, the local inflammatory reaction in response to chronic infection, even if mild, might also be an important factor to facilitate tumour development and it can also be a target in order to reduce its risk.

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