Master thesis

Rearrangement of the JC-virus genome

The transformation from a benign type to a PML virus

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Contents

Abstract

JC-virus (JCV) is present in approximately 90% of the human population and is the causative agent of progressive multifocal leukoencephalopathy (PML). JCV is usually present in the kidneys or urogenital tract and does not lead to disease or noticeable symptoms in healthy individuals. In certain cases however JCV may mutate from a benign viral type known as archetype to a malignant, PML causing type. This consequence is especially seen in HIV-infected patients and individuals undergoing immunosuppressive theory. We present two theories that may explain how this transformation may occur. Piling evidence exists that JCV influences DNA repair mechanisms in infected cells. Both agnoprotein and large-T protein have been shown to play an important role in this process. The extent of the influence on DNA repair suggests a more crucial role for this process than merely support of cell lysis. A close family member of JCV, SV40, also influences DNA repair mechanisms and hijacks cellular systems to recombine its genome. This supports the possibility that JCV may use a similar mechanism. Another theory regarding JCV genome recombination involves B-cells, which undergo VDJ-recombination when maturating. B-cells have been observed to be infected by JCV. Important support for this theory is that the genome of the PML variant of JCV contains duplicated Spi-B binding sites. Spi-B is a very important factor in maturating B-cells and is present in uniquely high levels in B-cells. It has been shown that DNA damage is also induced in lymphocytes, which links the DNA damage theory to the B-cell theory. Possibly JCV induces damage in B-cells, activating DNA damage repair mechanisms and simultaneously hijacks the VDJ-recombination to rearrange its genome and become a PML virus. We conclude that these two theories may both comprise part of the true mechanism.

Abbreviations

AGM	African Green Monkey kidney
AP	Agnoprotein
BKV	BK-virus
EBV	Eppstein-Barr virus
FDA	Food and drug administration
HRR	Homologous recombination directed DNA repair
IGF-1R	Insulin growth factor -1 receptor
IRS-1	Insulin receptor substrate -1
JCV	John Cunningham virus
MS	Multiple sclerose
NCCR	Non-coding control region
NHEJ	Non-homologous end joining
PML	Progressive multifocal encephalopathy
SV40	Simian virus 40
XLA	X-linked agammaglobulinemia

Introduction

In 1971 John Cunningham, a patient suffering from Hodgkins disease died from progressive multifocal leukoencephalopathy (PML) (Padgett *et al.,* 1971). PML is a rare neurodegenerative disease characterized by demyelination of (parts of) the central nervous system (CNS) as a result of destruction of oligodendrocytes (Darbinyan *et al.,* 2004). The three month mortality rate of PML is 20-50 % (Brew *et al.,* 2010). When brain tissue from John Cunningham was inspected with the electron microscope, large clusters of viral particles were seen in the abnormal areas of the brain. The virus was called John Cunningham virus (JCV) and appeared to be the causative agent of PML (Bellizzi *et al.,* 2013). JCV is a member of the polyomaviridae family.

Tests performed by several research groups indicate that 50 - 90 % of the human population is seropositive for JCV and that 19 - 27 % sheds infectious virus in their urine (Brew *et al.,* 2010). The mere presence of JCV does not automatically lead to the development of PML, however. In a healthy individual JCV is only present in the kidney tissue and occasionally the genitourinary tract and may therefore be excreted via the urine when the virus is actively replicating. This type of JCV can be considered the benign type. However when certain mutations occur these may enable the virus to migrate to the brain and cause lytic infection. JCV has then mutated to what can be considered as a very malignant type virus, with PML as a consequence (Marshall *et al.,* 2010, Gosert *et al.,* 2010).

Pathophysiology of PML development

There are several different risk factors identified for development of PML. These include several cancers; especially cancer types associated with the lymphoid system, long term immunosuppressive therapy and organ transplantation, because this may require chemotherapy or immunosuppressive therapy. HIV infected individuals are also at a higher risk to develop PML as well as elderly people, which may be a result of compromised cellular immune responses due to the process of aging (Ferenczy *et al.,* 2012). Immunosuppressive therapy however is currently applied as a ‘best option’ solution for several autoimmune diseases such as multiple sclerosis (MS) and Crohns disease and also following organ transplantation (Marshall *et al.,* 2010), therefore this is not a risk factor that is easily dismissed. The risk of developing PML that arises when applying immunosuppressive therapy however, is a very undesirable adverse effect and it is therefore essential to gain more knowledge regarding the mechanisms by which JCV mutates from a benign virus to a malignant PML virus.

The JCV genome

The JCV genome can be divided into three regions, the early and late region and a non-coding control region (NCCR) (fig 1). The early and late regions are highly preserved, whereas the NCCR is highly variable (Darbinyan *et al.,* 2004, Frisque *et al.,* 1984). The NCCR can be subdivided into six regions, boxes A to F. The benign type of JCV which is present in the kidneys, is also called archetype virus and has a linear distribution of boxes A to F. The standard malignant JCV type is named prototype virus and has a different line-up of boxes, from which boxes B and D have been deleted and boxes A, C and E have been duplicated. Besides the prototype there are numerous other variations of the malignant type (Gosert *et al.,* 2010, Ferenczy *et al.,* 2012, Marshall *et al.,* 2012). Despite the many malignant variants, the effect of the NCCR rearrangements is generally the same; viral promoting genome sequences such as transcription factor binding places important for viral resilience and cell specificity are duplicated and favorably positioned and viral repressing sequences are deleted (Marshall *et al.,* 2010).

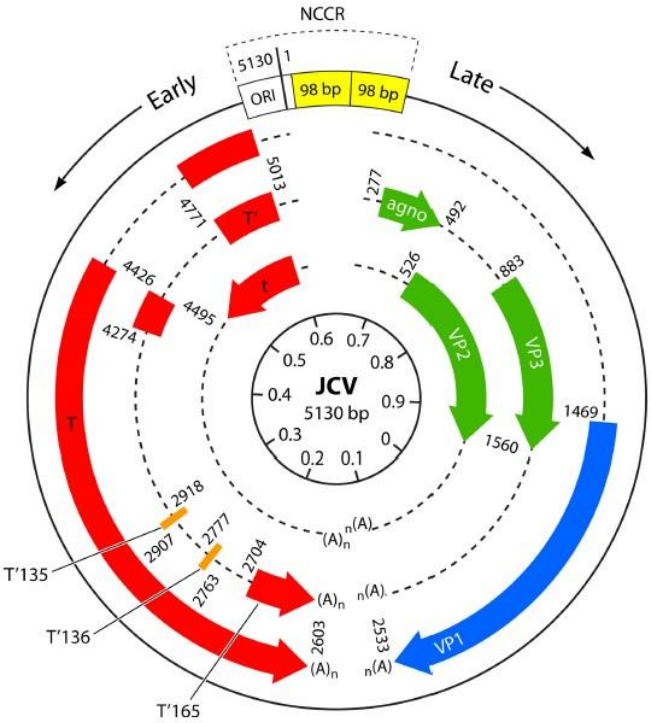

Figure 1 *JCV genome structure*
The JCV genome is visualized. In red are the early genes and in blue and green the late proteins. The early and late genes are transcribed in opposite directions (Swiss Institute of Bioinformatics, 2008).

Transmission & replication of JCV

The transmission of JCV is thought to occur via contaminated food and water. Studies regarding the global spread of JCV have shown that 98% of sewage samples tested was positive for JCV (Bofill-Mas *et al.,* 2001). No PML type virus was found in these samples however, indicating that the transmission from the benign archetype virus to the PML prototype virus always occurs in the infected individual and that this type is restricted to certain areas in the body because it is not shedded in the urine. A different possibility is that PML type virus is disseminated throughout the body but is not able to integrate. Early research in PML patients showed that in some PML patients the virus was present in the lungs, spleen, kidneys, lymph nodes and liver, however no evidence was found of viral DNA

integration into the cellular genome (Grinnel *et al.,* 1983). The presence of JCV in the lymph nodes and spleen may be explained by infected B-cells which may reside in these locations. The presence of JCV in the lungs and liver may possibly be the result of the absence of several factors required by PML type JCV to be able to integrate in the host genome and create progeny virus, for example members of the NF-1 protein family and a protein called Spi-B, both thought to play an important role in facilitating productive JCV infection (Marshall *et al.,* 2010).

Replication of JCV itself is dividable in two important stages, an early stage with early proteins, T antigen representing the most important early protein, and a late stage with late proteins which encode for capsid proteins and agnoprotein (fig 1), which is an important protein and will be discussed below. JCV is, as is common for polyomaviruses, dependent on host factors for replication. JCV replication is a relatively slow process, viral progeny is usually detected within three to five days and may continue for weeks. Whether it is required for JCV to integrate in the human genome or whether this may be different for either archetype or PML type virus is currently unknown (Ferenczy *et al.,* 2012). It would be interesting to conduct research regarding possible differences in replication style between archetype and PML type virus and to study whether they require different factors and need to be integrated in the genome to be able to produce progeny virus.

Theories regarding NCCR rearrangement

Exactly how and where JCV mutates from a benign virus to a malignant PML causing virus is currently unknown. Unfortunately the acquisition of this knowledge is restricted by several factors. JCV is oncogenic in laboratory animal models and due to its very strict species and cell specificity is therefore difficult to grow in culture (Darbinyan *et al.,* 2004).

Several theories regarding the mechanism JCV uses to become a PML virus have been suggested but none has been close to being proven yet. One of these theories with an interesting potential states that JCV may use the genome recombination mechanism in the maturation process of B-cells to ‘co-mutate’ and subsequently become a PML virus (Ferenczy *et al.,* 2012). Evidence exists that JCV is capable of infecting B-cells and this may possibly be the way of migrating to the brain (Tan *et al.,* 2009). The possibility that JCV uses the B-cell maturation process to mutate to a PML causing virus is discussed below.

A novel idea, which will be presented here, is to compare the recombination mechanism of Simian Virus 40 (SV40) to that of JCV. SV40 uses the DNA repair machinery in host cells to achieve recombination and form viral variants. BKV is a third member of the polyoma virus family and shows a high level of similarity to both JCV and SV40. BKV also rearranges its genome and is associated with severe disease (White *et al.,* 2009), although the relationship between the recombination and disease severity is sill debated. The exact mechanism by which BKV mutates into a pathogenic virus is also unknown, but perhaps by studying the recombination mechanisms of SV40 and comparing this to JCV and BKV the process of transformation may be elucidated.

Currently plenty of evidence exists that JCV influences host cell DNA repair and replication machinery, although it remains unclear how this is advantageous for viral replication. (Darbinyan *et al.,* 2004, Trojanek *et al.,* 2006A). It has not been investigated however if JCV could use the same machinery as SV40 to form variants and ultimately mutate to a PML virus. Therefore the parallels between SV40 genome recombination and JCV influence on DNA damage repair and the possibility that JCV may use a similar pathway will be considered here.

It is important to unravel the mechanisms behind the conversion of JCV considering the extremely poor prognosis of PML and the lack of measures to prevent the development of PML (Marshall *et al.,* 2010, Brew *et al.,* 2010). This review will focus on the current knowledge and hypotheses regarding the mechanism of conversion of the benign type of JCV, merely capable of infecting kidney and genitourinary tissue, to a malignant virus type capable of causing a lethal disease of the central nervous system.

Incitement of DNA repair mechanisms by JCV

Comparison of JCV, SV40 and BKV

The genome similarities between the three most important members of the polyomavirus family, JCV, SV40 and BKV are striking (fig. 2). All three generally contain the same structure, which includes important elements such as the highly preserved T-antigens and VP-proteins and the highly variable NCCR (White *et al.,* 2009). One of the main features of JCV and its ability to cause PML is that there is a significant difference in the structure of the NCCR between different viral strains. The benign archetypal variant of the virus contains boxes A to F in a linear distribution whereas the PML strains have a shuffled version of these boxes (Frisque *et al.,* 1984). The exact same phenomenon is seen in BKV, of which the archetype virus is also the benign transmittable type and has 5 boxes O, P, Q, R, S distributed

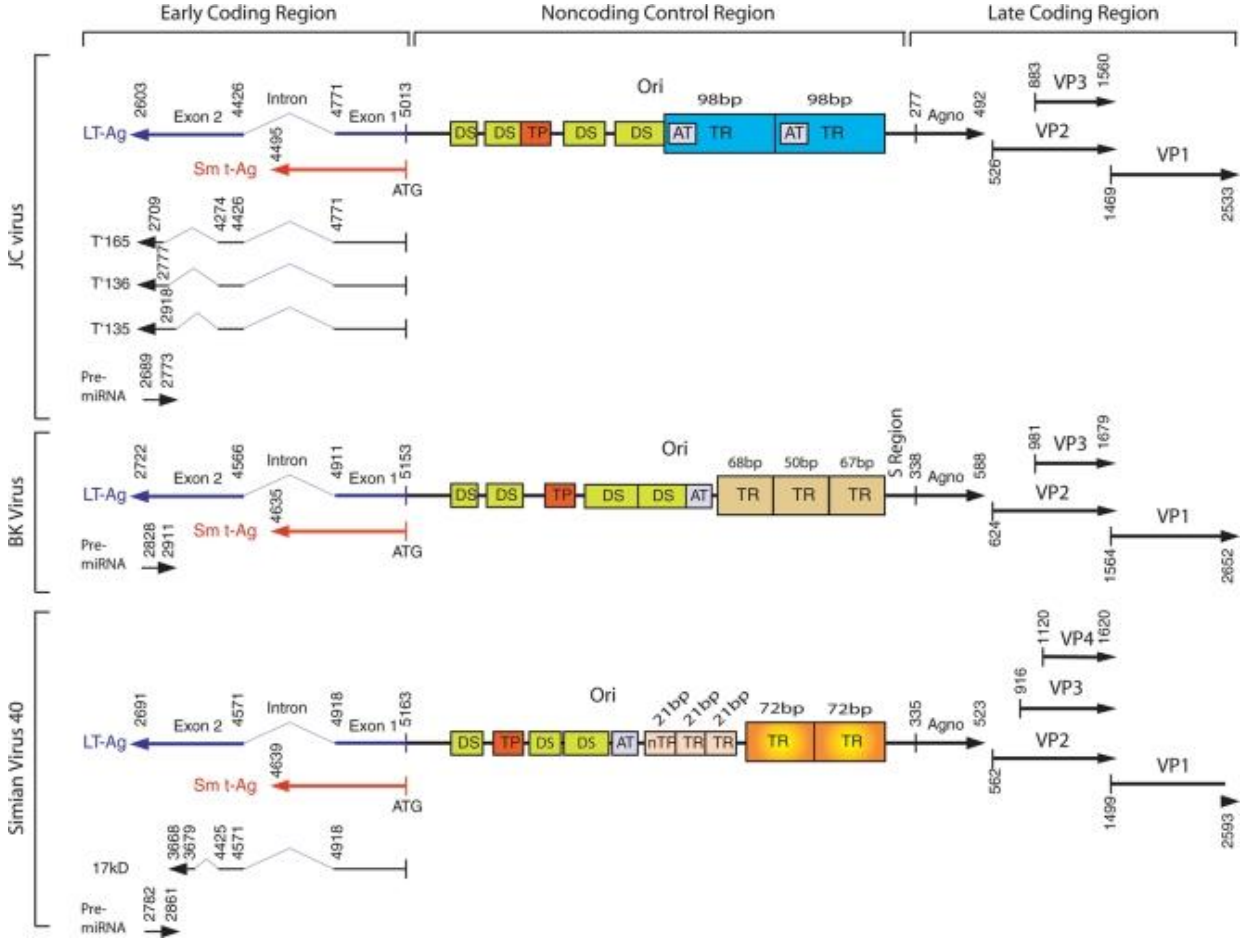

Figure 2 *Genome organization of JCV, SV40 and BKV*
The circular genomes of JCV, SV40 and BKV are visualized here in a linear schematic diagram. All three viruses show a high level of similarity in structure. On the left are the early coding regions, transcribed from right to left, on the right are the late coding regions, transcribed from left to right. In the middle are the NCCR's, which are highly variable between different viral strains, in contrast to the early and late regions which are highly preserved (White *et al.,* 2009).

in a linear fashion, but of which even a higher level of variety is seen in the mutated viral variants than of JCV NCCR variants (White *et al.,* 2009, Markowitz *et al.,* 1988). Similar to JCV, BKV archetype is also shed in urine (Yogo *et al.,* 2008). BKV mutations of the NCCR do not lead to PML however, but cause severe virus-associated nephropathy in humans (Gosert *et al.,* 2008, Hirsch *et al.,* 2005). SV40 does not cause PML in humans either, but is capable of causing this disease in the rhesus macaque monkey (White *et al.,* 2009). Just as is the case with JCV and BKV, the virus generally resides in the kidneys but may cause severe disease when mutations have occurred (Newman *et al.,* 1998).

The general feature of all three polyomaviruses is the rearrangement of the NCCR and subsequent pathogenesis of different severe diseases. Because there are so many similarities between these three viruses, it is also likely that a lot of viral processes to complete their replication cycle are conducted in the same manner. It is therefore a viable option to study a mechanism in one of the viruses with the aim to gain more knowledge about how another virus may succeed in a certain achievement.

The recombination mechanism of SV40

SV40, like JCV, is a member of the *polyomaviridae* family. It can therefore be assumed that they may use similar mechanisms in their reproductive cycle. In the case of genome recombination it is interesting to compare SV40 to JCV.

The mechanism by which SV40 inserts fragments into host DNA and forms variants was studied by Singer *et al.,* 2011. In 2007 the genome of the *Macaca Mulatta* was mapped and has become available for study. The *Macaca Mulatta* is a rhesus macaque monkey and closely related to the African Green monkey (Gibbs *et al.,* 2007). This provides the opportunity to compare SV40 recombined viral genomes to the genome of the *Macaca Mulatta* and study how and where SV40 has used this DNA to form its own variants. Using BSC-1 African Green Monkey kidney (AGMK) cells eight SV40 variants were analyzed. The locations of origin of the inserted monkey DNA were studied and this information was used to form a hypothesis regarding the recombination mechanisms used by SV40 (Singer *et al.,* 2011).

It was found that the inserted chromosomal DNA could either be linked to SV40 DNA or to other fragments of chromosomal DNA. Also out of the eight studied variants five variants contained more than one DNA insert in the same repeat unit derived from different chromosomal locations. The fact that monkey DNA from different chromosomal locations is present and that in some variants it is joined together provides information about how these variants may have arisen (Singer *et al.,* 2011) (fig 3). The event of so-called ‘capturing DNA’ is not unique to SV40, in yeast and mammalian genomes the insertion of extra chromosomal fragments of DNA has been observed before (Haviv-Chesner *et al.,* 2007, Little *et al.,* 2004). When the part of the SV40 genome that is inserted into the host genome contains an ORI, activation of this ORI would lead to the excision of this fragment together with part of the chromosomal DNA fragments attached to it. When this process is repeated several times

distinct new SV40 fragments would arise, linked to cellular DNA fragments from various chromosomal locations. When part of SV40 DNA which has previously been inserted and excised is inserted repeatedly in different areas of the host DNA, this may explain the presence of multiple inserts in the same repeat unit of the SV40 genome (Singer *et al.,* 2011).

It is interesting to study these events because insertion into the host DNA is not an essential step in the SV40 reproductive cycle but can be utilized to form new viral variants (Ferenczy *et al.,* 2012, Singer *et al.,* 2011). Evidence exists that JCV also influences DNA repair mechanisms in host cells and uses them for its own advantage (Darbinyan *et al.,* 2004, Trojanek *et al.,* 2006A). The use of these mechanisms by JCV to form new variants has not been demonstrated however. Since it has been proven that SV40 creates variants via this pathway (Singer *et al.,* 2011) it is interesting to consider the possibility that JCV may also use these mechanisms to form PML viruses, as we currently do not understand how JCV accomplishes this transformation.

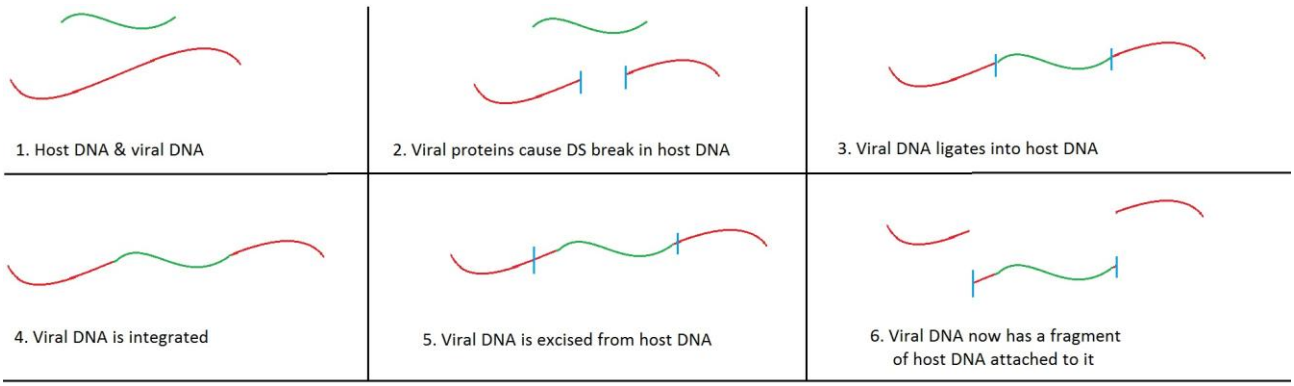

Figure 3 *SV40 insertion and excision into host DNA*
The mechanism used by SV40 to snatch fragments of host DNA with as a result the formation of new viral variants is visualized. All DNA is depicted as being single stranded for simplifying purposes. First the viral proteins cause a double stranded break in the host DNA. The viral DNA is then inserted into the host DNA and subsequently the breaks are repaired and the viral DNA has been integrated. Viral proteins then excise the integrated DNA. In this process parts of the host DNA may remain attached to the excised viral DNA. A new viral DNA fragment has arisen, consisting of viral DNA and host DNA.

Evidence that JCV may use DNA repair mechanisms for recombination

The first indication that JCV has any effect on the repair mechanisms of the infected cell is that a correlation has been found between JCV viral titers and increased chromosomal instability of the host cell (Lazutka *et al.,* 1996, M'Kacher *et al.,* 2010, Neel *et al., 1996,* Neel *et al.,* 1998). Peripheral blood lymphocytes of 44 individuals was tested for the presence of chromosome aberrations and their viral status regarding JCV and BKV was determined. It was found that in individuals the degree of chromosome aberrations correlated with the height of the viral titers for JCV and, less pronounced, the viral titers for BKV (Lazutka *et al.,* 1996). More recently a similar study was conducted in patients suffering from either

Hodgkins lymphoma or B-cell non-Hodgkins lymphoma. In this study it was observed that in patients positive for JCV the degree of chromosome aberrations was significantly increased, especially when co-infected with Eppstein-Barr virus (EBV). A correlation was found between the amount of JCV genome copy numbers per cell and the degree of chromosome aberrations. The exact interaction between JCV and EBV and why the combination of these viruses yields higher levels of genomic abnormalities is unclear and requires further investigation (M'Kacher *et al.,* 2010).

Research groups have also conducted *in vitro* studies to determine the effect of JCV in cultured lymphocytes. It appeared that under these circumstances the same effect was seen as in lymphocytes from studies discussed above; cells positive for JCV exhibited a significantly higher amount of chromosomal damage in comparison to healthy cells or cells exposed to minor radiation (Neel *et al., 1996,* Neel *et al.,* 1998).

The fact that there is an obvious relation between the presence of JCV and the occurrence of chromosome damage and genomic instability does not automatically lead to the irrefutable conclusion that JCV influences the DNA repair mechanisms, but does suggest a role for activating these systems by inducing DNA damage. The means by which JCV is suspected to influence DNA repair systems via several viral proteins is discussed hereafter.

Large T-influence on DNA repair mechanisms

Large-T is an early protein of JCV and is very important due to its many functions. It interacts with both viral and cellular proteins and DNA, particularly driving the host cell into S-phase, stimulating genome replication and participating in the process (Ferenczy *et al.,* 2012). A less extensively studied function of large-T is its influence on DNA repair systems of the host cell. It seems however that large-T has strong mutagenic abilities and in recent years the suspicion that it plays a role in the development of cancerous tumors has been growing (Trojanek *et al.,* 2006A, Trojanek *et al.,* 2006B, Reiss *et al.,* 2006, Reiss *et al.,* 2012, Vilkin *et al.,* 2012, Del Valle *et al.,* 2010). The exact influence of large-T on DNA repair mechanisms was investigated and a mechanism of action was proposed.

There are two major DNA repair mechanisms present in cells, non-homologous end joining (NHEJ) and homologous recombination directed DNA repair (HRR). The latter is considered to be more accurate than the first (Trojanek *et al.,* 2006A). For the functioning of HRR the insulin growth factor – 1 receptor/ insulin receptor substrate - 1 signaling axis (IGF-1R/IRS-1) is important (Trojanek *et al.,* 2003). When the repair mechanism is inactive, IRS-1 is bound to Rad51 in the cytoplasm. Upon stimulation of IGF-1, IRS-1 is phosphorylated and no longer bound to Rad51. Rad-51 binds to BRCA-2 and enters the nucleus, where it supports an adequate HRR response (Trojanek *et al.,* 2006A) (fig 4). Several studies have proven that the presence of large-T in a cell influences the functionality of the IGF-1R/IRS-1 signaling axis, though the exact mechanism by which large-T influences this axis and why this leads to genomic instability and mutations was not clear (Del Valle *et al.,* 2002, Sell *et al.,* 1993). More recently it appeared that the fact that large-T binds IRS-1 causes this complex to

translocate to the nucleus, where IRS-1 then reforms the complex with Rad51, attenuating its function in HRR and ultimately attenuating HRR itself. By inhibiting HRR the adequate repair of DNA damage is inhibited, leading to genomic instability (Trojanek *et al.,* 2006A).

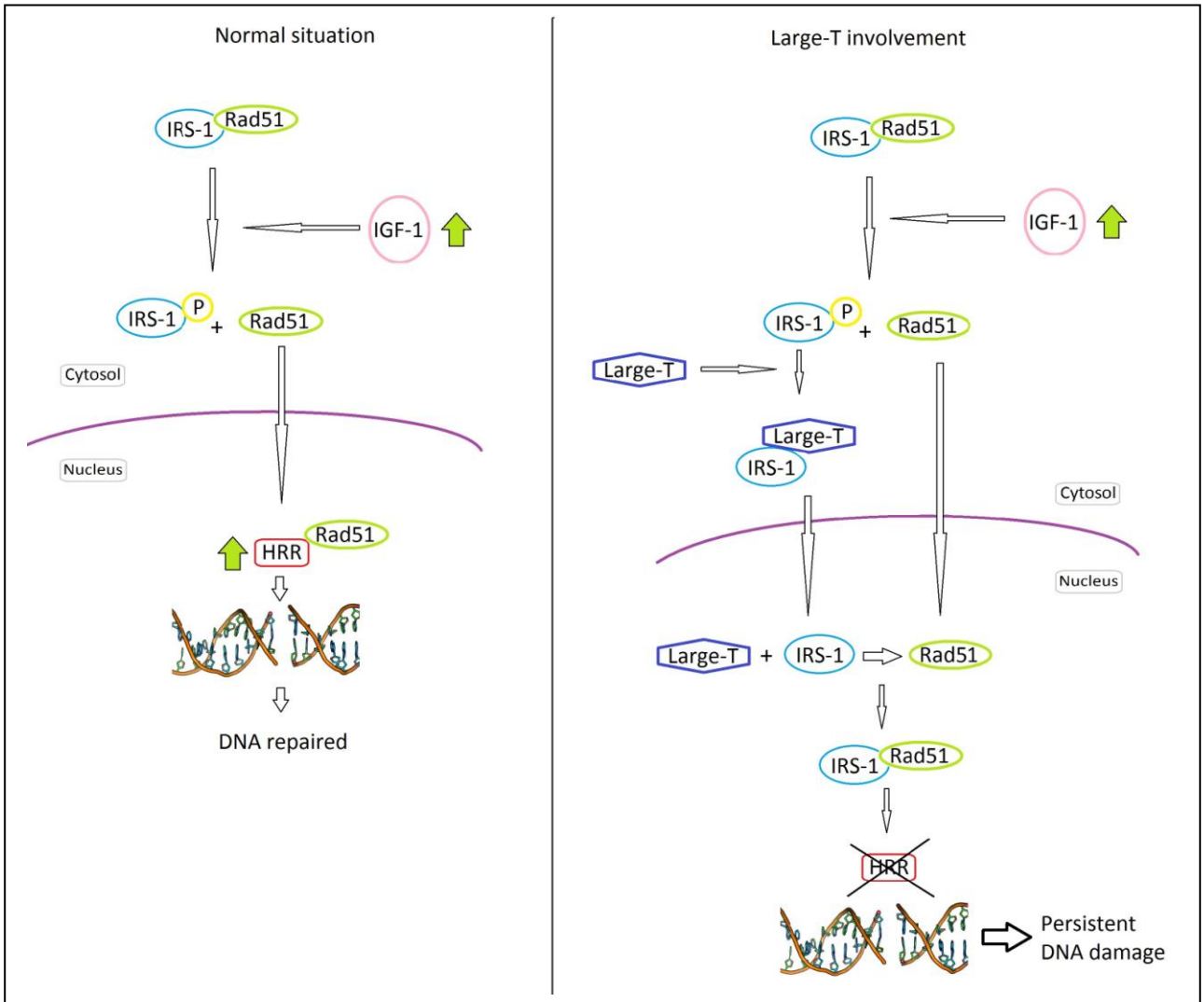

Figure 4 *The influence of JCV Large-T protein on homologous recombination directed DNA repair (HRR).* On the left the normal response to change in the IGF-1/IRS-1 axis as a result of DNA damage is visualized. IGF-1 stimulates the separation of IRS-1 and Rad51 and subsequent phosphorylation of IRS-1. Rad51 then translocates to the nucleus where it supports correct DNA repair. On the right the influence of Large-T on DNA repair is visualized. When IRS-1 and Rad51 are separated and Rad51 has translocated to the nucleus Large-T binds to IRS-1 and also translocates to the nucleus. IRS-1 and large-T disassociate and IRS-1 reforms the complex with Rad51, resulting in less efficient DNA repair due to the inactivity of Rad51. This attenuates adequate HRR and DNA damage persists (Trojanek *et al.,* 2006A).

These facts obviously show a relevant role for large-T in manipulating the cellular repair mechanisms and at least suggest a role in the development of cancerous cells and increased cell death. Whether this influence is also part of a greater picture in which JCV uses multiple ways to influence the repair mechanisms of the cell and ultimately uses these

systems for its own advantage in becoming a PML virus is currently unclear, but should be taken into account when considering this matter.

The role of agnoprotein

Agnoprotein is a late auxiliary viral protein of JCV and is not included in the JCV virion (Khalili *et al.,* 2005). There are several established and suggested roles for agnoprotein, for example the downregulation of viral gene expression and DNA replication (Safak *et al.,* 2001). Agnoprotein interacts with several cellular factors to accomplish its functions. One of its functions seems to be the induction of DNA repair mechanisms (. Darbinyan *et al.,* 2004, Khalili *et al.,* 2005).

Agnoprotein accomplishes the induction of DNA repair mechanisms by cooperating with the p53 protein, which is a known regulator of the cell cycle and a very important activator of DNA repair proteins (Alberts *et al.,* 5th edition). P53 and agnoprotein increase the amount of p21 protein in the cell, which is an inhibiting regulator of cell cycle progression at the G1 stage (Darbinyan *et al.,* 2002). The effect of the presence of agnoprotein in cells regarding their capability to respond efficiently to DNA damage and the influence of its presence on DNA repair mechanisms was studied (Fig 5). This was conducted by treating cells with cisplatin, which causes DNA adducts to arise and subsequently activate DNA repair mechanisms, including the p53 pathway. To begin with the difference in cell survival under the influence of cisplatin and the absence or presence of agnoprotein was studied. It was observed that cells expressing agnoprotein and treated with cisplatin had a significantly lower survival rate, indicating that the

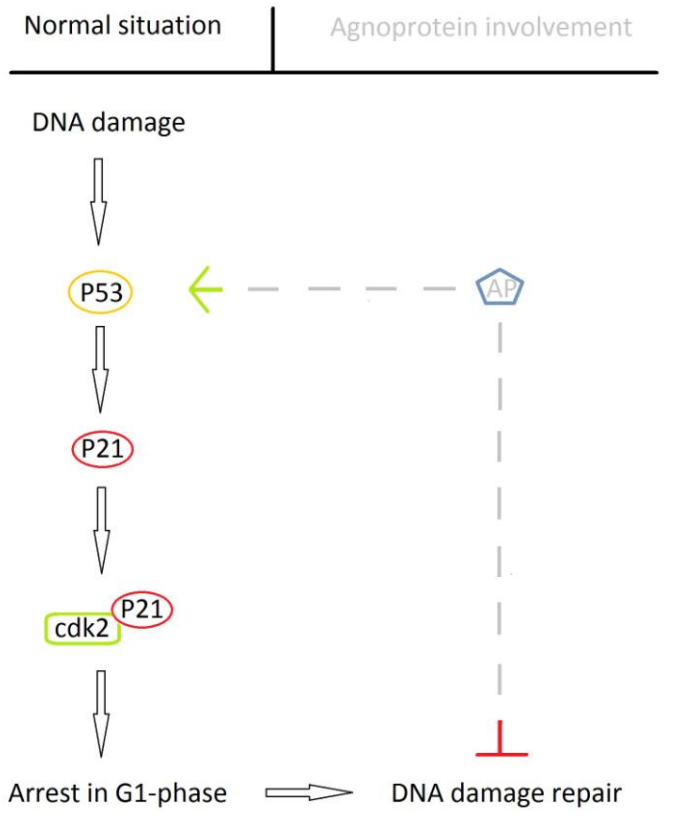

Figure 5 *Agnoprotein involvement in DNA damage repair.* In black the normal response to DNA damage in cells is visualized. In response to DNA damage P53 is activated, which in turn activates P21. P21 binds to cdk2 which causes the cell cycle to halt and reside in the G1 phase to enable DNA damage repair, before continuing into S-phase (Vogelstein *et al.,* 2000). In grey the situation involving agnoprotein (AP) is visualized. Agnoprotein stimulates P53 to activate P21 and subsequently halt the cell in G1-phase (Darbinyan *et al.,* 2002). Additionally agnoprotein seems to impair the correct functioning of the DNA damage repair mechanism. Exactly how this is achieved is unclear (Darbinyan *et al.,* 2004).

presence of agnoprotein decreases the ability to responds adequately DNA damage. Direct evidence of DNA damage was also seen, the genomes of cisplatin+/agnoprotein+ cells displayed extensive abnormalities in chromosome structure and severe chromosomal fragmentation, whereas cells treated with cisplatin but not expressing agnoprotein only showed mild chromosome damage and a reasonable repair capability (Darbinyan *et al.,* 2004). These well-defined effects of agnoprotein and other elements of JCV seem to indicate that influencing the DNA repair capacities of infected cells is very important. Because JCV seems so have several different systems to accomplish this effect it seems viable to hypothesize that this influence does not function as merely an aid to cell lysis, but also as a possible key point in taking over the cellular DNA repair mechanisms to mutate the JCV genome and become a PML virus.

The use of DNA repair mechanisms to form JCV variants

The evidence above clearly shows that JCV influences DNA repair mechanisms of the infected cells in several ways (Fig 6). Agnoprotein stimulates P53 to halt the cell cycle in the G1-phase and additionally inhibits the repair of DNA damage (Darbinyan *et al.,* 2002, Darbinyan *et al.,* 2004). Large-T protein inhibits DNA damage repair by translocating IRS-1 to the nucleus, where it forms a complex with Rad51, attenuating HRR and leading to persistent DNA damage (Trojanek *et al.,* 2006A). The fact that multiple proteins are used (possibly other unknown proteins as well) to manipulate the cells' machinery suggests that this is a very important feat in the JCV life cycle. In earlier research it was already suggested that this manipulation functions as a lytic mechanism to release new virions and subsequently expose other cells to the virus (Darbinyan *et al.,* 2004).

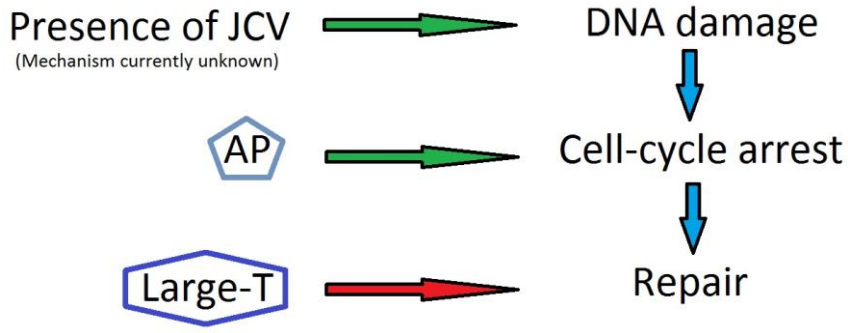

Figure 6 *General influence of JCV*
A short summary of JCV influences on infected cells is visualized. In cells infected with JCV evident chromosomal damage and instability is observed. Agnoprotein (AP) acts via the P53 pathway to halt the cell cycle and inhibits DNA damage repair. Large-T also inhibits DNA damage repair by influencing the IGF-1/IRS-1 axis and ultimately attenuating HRR. As can be concluded from this figure, the general influence of JCV results in a cell with genomic damage, halted in G1-phase, but without a functioning repair mechanism.

The use of maturing B-cells as a genome recombination mechanism

A different but potentially related theory regarding the rearrangement of the JCV NCCR involves the use of immature B-cells to aid the recombination and formation of viral variants, including viruses capable of causing PML. In B-cell maturation an interesting mechanism is seen. To be able to provide numerous variations of important proteins of the immune system, the immunoglobulins, the B-cells recombine their genomes to form novel variations of immunoglobulins (Kuby *et al.,* 6th edition). It has been observed and discussed above that JCV has an influence on the genomic instability of lymphocytes, a phenomenon that has also been seen in B-cells (M'Kacher *et al.,* 2010, . Trojanek *et al.,* 2006A). It has also been suggested that B-cells transport JCV across the blood-brain barrier (Ferenczy *et al.,* 2012). We here explain a theory that JCV may use the recombination system used by B-cells to recombine its genome and form new viral variants capable of causing PML.

VDJ recombination in B-cells

Immunoglobulins are an extremely important part of our immune system. They are part of the humoral segment of the adaptive immune system and their function is to neutralize and eliminate foreign substances from the body, such as viruses and bacteria (Robbins & Cotran, 8th edition). Because the variation in antigenic intruders is limitless, the human body must adapt to this variety and be able to supply a limitless variability in immunoglobulins. They are produced by B-cells, using a very elegant system to ensure an infinite number of distinct immunoglobulins. The B-cell maturation process starts in the bone marrow. Here the genome of these immature cells is recombined to ensure genetic variability and therefore the ability to produce novel immunoglobulins. This process is called VDJ-recombination (Kuby *et al.,* 6th edition). This mechanism includes several important events, the V-, D-, and J- segments are recombined, nucleotide deletions on the 3'-end take place, nucleotides are inserted and added and mutations are induced. All these events together generate a extensive diversity in immunoglobulins (Li *et al.,* 2004).

It has been suspected for some time that JCV uses B-cells to travel to the human brain and cause PML. There is growing evidence however that B-cells do not merely enable migration to the brain, but also provide a site of recombination of the JCV genome, enabling it to mutate from a benign virus to a malignant, PML causing virus (Marzocchetti *et al.,* 2008, Tan *et al.,* 2009, Ferenczy *et al.,* 2012, Jensen *et al.,* 1999).

JCV infection rate in B-cells

It is important to study how much B-cells are or can be infected by JCV in an individual, for this is crucial information to determine whether B-cells are a possible site for JCV to mutate into a PML virus. However exact data regarding the amount of infected B-cells in an individual is difficult to appoint due to the fact that this has not been thoroughly studied. Several groups have researched the overall presence of JCV in B-lymphocytes and other locations in the human body. Chiara *et al.,* tested 54 tonsillar tissue samples from both adults and children. Approximately 37% of all samples was positive for JCV DNA, tested via either T-protein primers or regulatory region primers. Both archetype and PML-type virus were identified and these researchers therefore suggest the tonsils as a location for primary infection with JCV (Chiara *et al.,* 1998). Another research group summarized a number of different studies regarding JCV infection in lymphocytes in particular, and found that out of a total of 507 patients, 156 individuals were positive for JCV. For a full review regarding the nature of cells in which JCV infection was found and presence of underlying conditions see Gallia *et al.,* 1997.

Factors influencing JCV tropism

Research has shown that in a number of JCV susceptible cells certain specific cellular factors are present in relatively higher levels than in non-susceptible cells. Two notable factors are Spi-B and NF-1X (Ferenczy *et al.,* 2012). NF-1X is a member of a protein family consisting of four members, A, B, C and X, sometimes also known as D in literature. All four are DNA binding proteins. They function in gene transcription and have been observed to play a role in the replication of several viruses, including JCV (Sumner *et al.,* 1996, Monaco *et al.,* 2001). The JCV genome several has Spi-B binding sites in its promoter/enhancer sequences, which are actively used by the PML causing variants of the virus (Major *et al.,* 2010B). Strikingly, Spi-B is also involved in B-cell maturation. The upregulation of this protein therefore links JCV infection to B-cell maturation (Marshall *et al.,* 2012).

It was found that high expression of NF-1X was correlated with the susceptibility of cells for JCV. Human fetal glial cells with high NF-1X expression were susceptible, whereas HeLa cells with low NF-1X but high NF-1C expression were non-permissive for JCV infection (Sumner *et al.,* 1996). The relative levels of different NF-1 types seem to influence the susceptibility for JCV. For example it was found that kidney and epithelial cells, in which the benign type of JCV resides, also express low levels of NF-1X (Apt *et al.,* 1994). Possibly the PML causing variants of JCV are more dependent on NF-1X levels, explaining why infection with these viral variants does not occur in the kidneys but does occur in B-cells and brain cells.

Spi-B is a transcription factor which is mainly present in B-cells. It is essential for proper B-cell receptor signaling and is important in the correct formation of germinal centers in the spleen (Garrett-Sinha *et al.,* 1999). Spi-B plays an important role in the correct

differentiation and maturation of B-cells and is expressed in high quantities in maturing B-cells (Su *et al.,* 1996, Ray *et al.,* 1992).

Early studies reported Spi-B to be exclusively expressed by B-cells, this has been refuted however by more recent research applying more sensitive measuring techniques. It was observed that other JCV susceptive cell types also express Spi-B, though in lesser quantities. The expression of Spi-B was measured in brain derived SVG cells, CD34+ lymphocytes and the haematopoietic precursor cell line KG-1a, and was compared to Spi-B expression in several B-cell lines. It was observed that in these cells relatively lower levels of Spi-B is being expressed. In the same study it was also seen that in JCV variants capable of causing PML there were more Spi-B binding sites present in the NCCR that actively bound Spi-B, whereas in the benign archetype NCCR this was not the case (Major *et al.,* 2010B). This suggests an important role for Spi-B in the pathogenesis of JCV-PML. Moreover, Spi-B has also been noted to stimulate gene expression from SV40, a family member of JCV (Petterson *et al.,* 1987). The exact influence of Spi-B on cell susceptibility was studied. Spi-B and NF-1X expression was induced in non-permissive progenitor-derived neurons and later these cells were infected with a PML-variant of JCV. Five days post infection a significant expression of T-antigen was observed, showing that non-permissive cells can be 'made' to be susceptible to JCV infection (Major *et al.,* 2010B). The fact that after induced expression of Spi-B in a non-permissive cell infection with JCV is possible indicates the significant importance of Spi-B binding sites on the ability to replicate in a specific cell type.

The effect of natalizumab on the development of PML

Natalizumab is a humanized monoclonal antibody, used to treat several autoimmune diseases including multiple sclerosis and Crohn's disease. Natalizumab acts by binding to α-integrins on T-cells and B-cells, preventing migration into the gut, brain and epithelia (Krumbholz *et al.,* 2008). In 2005 the use of this drug was linked to two cases of PML and was therefore withdrawn from the market (Kleinschmidt-DeMasters *et al.,* 2005). One year later however the U.S. food and drug administration (FDA) reapproved the drug but added a warning regarding the low, but present risk of developing PML (Lindberg *et al.,* 2008). The incidence of PML as a result of natalizumab treatment is estimated to be 3,85 per 1000 patients (Ferenczy *et al.,* 2012). The effect of natalizumab on B-cells and how this drug could be related to the development of PML was investigated.

It was observed that natalizumab significantly increases the amount of circulating pre-B-cells and B-cells in the periphery, automatically increasing the amount of susceptible cells to be infected by JCV, because of their high Spi-B levels due to maturation processes. Thus the mobilization of these B-cells creates a more widespread field for JCV to infect cells, mutate to a PML virus and migrate to the brain, causing PML (Major *et al.,* 2010A, Krumbholz *et al.,* 2008).

Discussion

Currently the mechanism that JCV uses to mutate from a benign virus, present in approximately 90% of the human population, to a malignant virus that causes the rapidly lethal disease PML is not known. JCV has a very strict tropism and host specificity, which makes it very difficult to conduct research. This is one of the reasons there is so little information available regarding the mechanism JCV uses to form new viral variants and ultimately migrate from the urinary tract to the brain and cause PML. Here we present two theories that may explain how JCV transformation occurs.

One method to unravel mechanisms used by viruses is by studying their closest family members. SV40 and BKV are both close family members of JCV and they indeed share many mechanisms. Both KBV and SV40 contain a highly variable NCCR which enables them to form numerous variants, every one possessing a different degree of ability to cause disease to an organism. New variants of SV40 are formed when the viral genome is inserted into the host genome, which is subsequently copied and excised. In this process it is possible that fragments of the host genome are snatched and inserted in the genome of SV40.

To be able to insert viral DNA into the host genome, the host genome must be damaged to create an opening and afterwards must be repaired to allow duplication. SV40 must therefore possess a mechanism to either induce DNA damage or influence DNA damage repair mechanisms, with the objective to accomplish the insertion of viral DNA, or to activate repair mechanisms to repair the DNA while viral and host DNA are being ligated together. Evidence shows that JCV also influences the DNA repair mechanisms of the host cell. Both large-T protein and agnoprotein have established role in the dysregulation of DNA repair mechanisms and cause cells to accumulate in the G1-phase of the cell cycle, unable to proceed into S-phase. Cells infected with JCV also show a significant inability to respond adequately to DNA damage, leading to chromosomal damage and genomic instability. Earlier researchers have suggested the manipulation of host cell DNA repair mechanisms as an important process to accomplish cell lysis and subsequent release of new virus. However the expanse of the viral abilities to influence the repair mechanisms is such that one might suspect another function of this influence. I suggest that this extensive influence not only functions as lytic support but also as a mechanism of rearrangement of the JCV genome. JCV's close family member SV40 also uses the cellular machinery to rearrange its genome and form variants. Thus far this potential mechanism has not been investigated for JCV. Considering the similarities between JCV, SV40 and BKV on all kinds of different aspects of their replication cycle and the fact that there is clear evidence that JCV influences DNA repair mechanisms, it seems very likely that it is possible that JCV also uses this system to become a PML virus.

Another theory regarding the rearrangement of the JCV genome lies with B-cells. Research has shown that approximately 30% of patients with different underlying diseases have B-cells that are positive for JCV and that in 37% of tonsillar tissues retrieved from 54

individuals JCV DNA was present, both archetype and PML-type. These immune system lymphocytes use VDJ-recombination to rearrange their genomic information to be able to form an infinite number of unique immunoglobulins. This system is active while these cells are maturing from pre-B-cells to mature B-cells. In this period a high level of Spi-B, a transcription factor, is present. Spi-B seems to play an important role in the susceptibility of cells for JCV infection. When assuming for a moment that JCV requires the genome recombination mechanism in B-cells to mutate to a PML-causing virus, the importance of Spi-B for the JCV replication cycle may be explained. It is possible that the strong dependence on the presence of Spi-B in a host cell is a selective advantage, because this is the one factor that is very specifically present in high levels in cells that are actively recombining their genome: the maturing B-cells. Therefore, by ensuring the need for Spi-B, JCV ensures that only cells that are recombining their genome are infected by the virus and that JCV can use their recombination system to mutate its own genome. The consequence of the fact that other cells also express Spi-B in lower quantities is that the recombined JCV variants can also infect these cells and replicate here. Because these other Spi-B expressing cells are cells present in the brain, the result is PML.

Of course, the reality of the actual mechanism used by JCV may lie somewhere in the middle of these two suggested theories. JCV could infect maturing B-cells, cause damage to the genome and subsequently influence the DNA repair mechanisms, while at the same time the recombination system is active which can then be hijacked by JCV to be used to rearrange the viral genome and form variants. This possibility is supported by the findings that DNA repair systems were particularly affected in peripheral blood lymphocytes, a group that includes B-cells. It may therefore be possible that JCV uses a combination of both influencing the DNA repair mechanisms and the VDJ recombination system in the same B-cell to achieve NCCR rearrangements and the formation of novel viral variants.

In 2003 a case report was published regarding a patient suffering from congenital x-linked agammaglobulinemia (XLA) who died from the consequences of PML. This is a unique case because this patient did not undergo any means of immunosuppression and was not infected with HIV or any other pathogen related to PML, save for JCV. It seems that PML has developed in this individual despite the severe impairment in B-cell differentiation and B-cell functioning. This case contradicts the hypothesis that (maturing) B-cells are required to develop PML. However the impairment of B-cells in XLA is not 100% and the residual amount of functioning B-cells present in the blood of this patient was not measured (Teramoto *et al.,* 2003). The fact that this is the only account of a patient developing PML while suffering from a B-cell impairing condition may even promote the possibility that B-cells are in fact required for PML development and that the existence of this unique case is simply possible due to a residual functioning of some B-cells which have facilitated the mutation process of JCV to a PML virus.

To provide evidence for the novel theory discussed here several things have to be studied extensively. The effect of JCV presence on genome stability in maturing B-cells would have to be studied to see in what measure this would be affected and how well the cells are

capable of repairing the damage. This could be tested in several different cell lines, like the haematopoietic progenitor cell line KG-1A or the BJAB cell line, an immortalized B-cell line capable of performing VDJ-recombination (Major *et al.,* 2010B). It would ultimately be necessary to test whether immature B-cells infected with archetype JCV are capable of producing PML virus when maturation is induced. If this can be demonstrated it would be proof that immature B-cells are capable of harboring archetype JCV virus and accommodating the transformation to a PML virus. When screening for both archetype and PML type virus in these cells it is important to monitor the possible intermediate viral genomes by sequencing methods. It has been shown that both archetype and PML type can be found in these cells, therefore if the transition from archetype to PML actually does occur in these cells the intermediate types which are neither a 100% archetype nor 100% PML type would be present and detectable. This would substantiate the hypothesis that transformation occurs in these cells.

In addition tests would have to be performed in cells that are not capable of performing VDJ recombination, the human fetal brain-derived SVG cell line for example, to see whether JCV could be coaxed into transforming into a PML virus. If both maturing B-cells and non-VDJ cells would be shown to produce PML virus this proves that JCV may mutate to a PML virus via several different pathways. It should then be investigated what the added advantage of using the VDJ system in B-cells is to becoming a PML virus or whether there is none and B-cells are simply just another cell type which is capable of harboring JCV and facilitating replication. However the relatively rare occurrence of PML suggests that JCV does require some very specific circumstances and factors to become a PML virus, therefore requiring the presence of a system like VDJ recombination for mutation in host cells seems attractive.

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