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THESIS

HUMAN POLYOMAVIRUS BK AND MOLECULAR ACTORS IN PROSTATE CANCER DEVELOPMENT: RESULTS, QUESTIONS, PERSPECTIVES OF A FOUR YEAR LONG STUDY.

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INTRODUCTION

The human prostate is a small walnut-sized composite organ composed of three glandular zones and a fourth non glandular region termed the anterior fibromuscular stroma which are best demonstrated by examination of cut sections in the sagittal, coronal and oblique coronal planes. These different zones are tightly fused together within a common sheath of fibromuscular tissue: the 'capsule' (Fig.1), (Mc Neal, 1968). The prostate wraps around the urethra and its function is to secrete a slightly alkaline fluid that usually constitutes 20–30% of the volume of the semen along with spermatozoa and seminal vesicle fluid. Prostatic fluid have better motility, longer survival and better protection of the genetic material. Prostatic secretions vary among species. They are generally composed of simple sugars and are often slightly alkaline. In human prostatic secretions, the protein content is less than 1% and includes proteolytic enzymes, prostatic acid phosphatase, and prostate-specific antigen. The secretions also contain zinc that is more concentrated (500–1000 times) than in blood. To work properly, the prostate needs male hormones, mainly testosterone, which are responsible for male sex characteristics (Huggins et al., 1942).

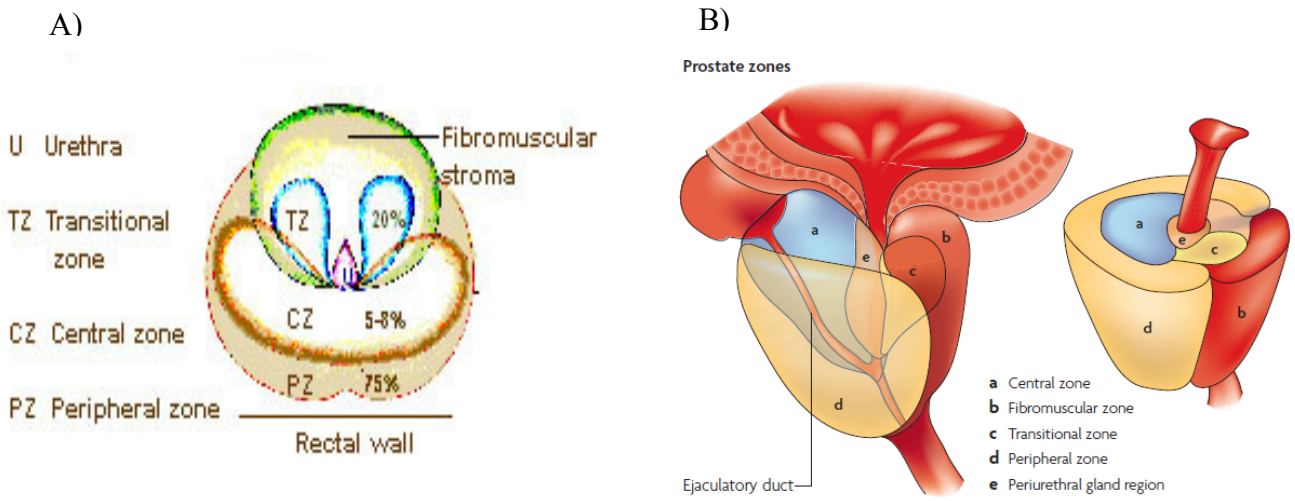

Figure 1: Schematic representation of the prostate. A) Romer et Parsons. The Vertebrate Body. 1977. Philadelphia, PA: Holt-Saunders International. p 395; B) De Marzo et al. Nat Rev Cancer. 2007; 7(4):256-69.

Disorders that could affect prostate are prostatitis, benign prostatic hyperplasia (BPH) and prostate cancer (PC).

Prostatitis is an inflammation of the prostate gland. There are four different forms of prostatitis: acute prostatitis, chronic bacterial prostatitis, male chronic pelvic pain syndrome and a rare type of leukocytosis.

BPH occurs in older men, the prostate often enlarges to the point where urination becomes difficult and if the gland grows too large, it may constrict the urethra preventing the flow of urine and making urination difficult and painful and, in extreme cases, completely impossible. BPH can be treated with medication, a minimally invasive procedure or, in extreme cases, surgery that removes the prostate. Minimally invasive procedures include transurethral needle ablation of the prostate (TUNA) and transurethral microwave thermotherapy (TUMT) (Lee et al., 2011).

Prostate cancer is the most frequently diagnosed cancer other than skin cancer and the second leading cause of death from cancer in men in the United States (Siegel et al., 2011). In Italy, the incidence trend showed a steep increase all over the country during 1970-2005 with a more pronounced increase in the Center-North then in the South of Italy. Incidence of northern and central regions was about twice as high as that of southern regions. Mortality trends were however constant or declining in the majority of northern-central regions whereas they still increased in the South. A total of around 43,000 incident cases, 174,000 prevalent cases and 9,000 deaths were estimated for Italy in 2005 (Inghelmann et al., 2007).

Early prostate cancer usually causes no symptoms. Sometimes, however, prostate cancer cause symptoms often similar to those of diseases such as BPH. The main symptoms are: frequent urination, nocturia, difficulty starting and maintaining a steady stream of urine, hematuria and dysuria. Finally, since the *vas deferens* deposits seminal fluid into the prostatic urethra and secretions from the prostate gland itself are included in semen content, prostate cancer may also cause problems with sexual function and performance, such as difficulty achieving

erection or painful ejaculation (Miller et al., 2003). Advanced prostate cancer can spread to other parts of the body possibly causing additional symptoms. The most common symptom is bone pain, often in the vertebrae, pelvis or ribs. Spread of cancer into other bones such as the femur is usually to the proximal part of the bone. Prostate cancer in the spine can also compress the spinal cord causing leg weakness and urinary and fecal incontinence. (Van Der Cruijsen-Koeter et al., 2005).

The rationale for screening is that early detection and treatment of asymptomatic cancers could extend life as compared with treatment at the time of clinical diagnosis. Effective cancer screening requires an accurate, reliable and easy-to-administer test that detects clinically important cancers at a preclinical stage and the availability of effective treatment that results in better outcomes when administered early rather than after signs or symptoms of disease have developed.

Prostate cancer diagnosis and treatments

A common initial strategy is Watchful Waiting also called *active surveillance*. This involves monitoring the tumor for signs of growth or the appearance of symptoms. The monitoring process may involve blood tests, manual palpation of the prostate or repeated biopsies. The goal of surveillance is to avoid overtreatment and, sometimes, serious, permanent side effects of treatment for a slow-growing or self-limited tumor that would never cause any problems for the patient. Active surveillance is the best choice for older, low-risk patients (Albertsen, 2011).

For many years, the digital rectal examination was the primary screening test for prostate cancer. However, this test has considerable interexaminer variability and the majority of cancers detected by means of digital rectal examination are at an advanced stage (Hoffman, 2011). In the late 1980s, PSA testing, which was initially developed for prostate-cancer surveillance, was rapidly and widely adopted for screening. Initially, PSA values above 4.0 ng per milliliter were considered abnormal though lower cutoff levels have subsequently been proposed.

Yet, most abnormal PSA values are false positive results that can be caused by BPH, prostatitis or cystitis, ejaculation, perineal trauma or the recent use of instruments for testing or surgery in the urinary tract. Numerous approaches have been proposed to improve the diagnostic accuracy of the PSA test, including measuring PSA velocity (change over time), levels of free and protein-bound PSA, PSA density (the PSA level divided by the prostate volume), and the use of cutoff values for PSA levels that are specific to the patient's age and race or ethnic group (Greene et al., 2009). However, the clinical usefulness of these strategies remains unproved.

Ultrasound scan can aid diagnosis showing the exact size of the prostate and allows to distinguish BHP from cancer, nevertheless, the definitive test is done by taking a biopsy. When a tumor is found, tumor biopsies are graded by Gleason grading and TNM classification. Gleason grading grades tumors on a scale of 1-5. Pattern 1 corresponds to a cancerous prostate closely resembles normal prostate tissue whereas pattern 5 corresponds to tissue does not have recognizable glands (Table 1). TNM classification is based on description of tumor size (T), lymph nodes involvement (N) and presence of distant metastasis (M) (Table 2).

Finally Computed Tomography (CT) scan and bone scan may be done to determine whether the cancer has spread (Stamatiou 2011).

Based guidelines for prostate cancer can guide treatment choices for specific clinical situations. The selection of treatment options involves many factors. For example, if radiation therapy is done first and fails, then radical prostatectomy is a very technically challenging surgery and may not be feasible. However therapies for aggressive prostate cancers involves surgery (i.e. radical prostatectomy), radiation therapy, high-intensity focused ultrasound (HIFU), chemotherapy, oral chemotherapeutic drugs, cryosurgery, hormonal therapy or some combination (Heidenreich et al., 2008).

Table 1: The Gleason's score/system for histologic grading of prostate cancer

GLEASON PATTERN	GLEASON SCORE	HISTOLOGICAL GRADE	DIFFERENTATION
1, 2	2, 3, 4	well differentiated	1
3	5, 6	moderately differentiated	2
4, 5	7, 8, 9, 10	poorly differentiated	3

(National Cancer Institute)

Table 2: The TNM system for staging of cancer

STAGE	TUMOR SIZE	LYMPH NODE INVOLVMENT	METASTASIS PRESENCE
Occult carcinoma	TX	N0	M0
Stage 0	Tis*	N0	M0
Stage I A	T1 a,b	N0	M0
Stage I B	T2 a	N0	M0
Stage II A	T1 a,b	N1	M0
	T2 a	N1	M0
	T2 b	N0	M0
Stage II B	T2 b	N1	M0
	T3	N0	M0
Stage III A	T1, T2	N2	M0
	T3	N1, N2	M0
	T4	N0, N1	M0
Stage III B	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1, a, b

***Tumour in situ**

(National Cancer Institute)

Prostate cancer aetiology

PC, as all type of cancers, is a multifactorial pathology and several risk factors contribute to its development. The primary risk factors are age and family history. PC is very uncommon in men younger than 45 but becomes more common with advancing age. The median age at diagnosis is 67 years and the median age at death is 81 years (Hoffman 2011). For unknown reasons, prostate cancer is more common in African-American men than in men of other races. African-American men are also more likely to have a more advanced disease when it is found and are

more likely to die of the disease. Prostate cancer occurs less often in Asian-American and Hispanic/Latino men than in non-Hispanic whites. The reasons for these racial and ethnic differences are not clear (Chornokur et al., 2011).

The androgen receptor (AR) is a type of nuclear receptor that is activated by binding of either the androgenic hormones testosterone or dihydrotestosterone. Low serum testosterone levels in men with newly diagnosed and untreated prostate cancer have been found to correlate with higher AR expression, increased capillary vessel density within the tumor and higher Gleason score. AR expression is observed in primary prostate cancer and can be detected throughout progression in both hormone sensitive and hormone refractory cancers. Although animal models of prostate cancer have suggested that elevation of AR expression can initiate prostate cancer development or is associated with recurrent growth in the presence of low androgen, the persistent heterogeneity of human prostate cancer suggests that increased AR expression is not generally associated with prostate cancer initiation and that hormone refractory prostate cancers are not clonally selected from AR-negative foci. The cause of the loss of AR expression in some cells of tumor foci is unclear. X chromosome losses, including loss of the AR gene, are extremely rare in prostate cancer. Epigenetic silencing of AR expression by methylation may occur and has been observed in 8% of primary prostate cancers (Heinlein et Chang 2004). AR mutation is hardly detectable in localized primary prostate cancers but is detected in about 10–30% of the hormone refractory cases and metastases of prostate cancer (Marques et al., 2011). Another possibility for the loss of AR expression in some tumor cells is a decrease in AR protein stability that reduces the AR protein level to one difficult to detect immunohistologically (Heinlein et Chang 2004).

Genetic background may contribute to prostate cancer risk, in fact, men who have a first-degree relative (father or brother) with prostate cancer (PC) have twice the risk of developing the disease and those with two first-degree relatives affected, have a fivefold greater risk compared with men with no family history (American

Cancer Society, 9 August 2010). Studies of twins in Scandinavia suggest that forty percent of prostate cancer risk can be explained by inherited factors (Lichtenstein et al., 2000). However, genetic alterations can also be induced by endogenous and exogenous carcinogenic factors as in most sporadic cancers. For practical purposes, prostate cancers can be divided into three groups: hereditary, familial and sporadic. Hereditary prostate cancer is defined as nuclear families with 3 cases of prostate cancer, Familial PC refers to families with cancer in each of 3 generations and/or with 2 men diagnosed before age 55 years. Sporadic prostate cancer is defined as patients with no family history (Siddiqui et al., 2006).

As mentioned above, carcinogenic factors can be endogenous (i.e. reactive radical species) or exogenous (i.e. ultraviolet A rays, oncogenic viruses), however, all factors altered oncogenes and/or tumor suppressor genes in the cells. In PC a great number of genes have been implicated and investigated, nevertheless, in this study MYC gene, BIRC5/Survivin gene, CDC25 gene and P53 gene were considered.

The MYC oncogene (8q24) is an example of a gene with copy number gain but without sequence alterations in prostate cancer. MYC mRNA was found to be elevated in the cancer tissue as compared to matched benign prostatic tissue in the majority of cases. Highly consistent upregulation of MYC at the mRNA level was seen in the majority of prostate cancers across a large number of patient samples from multiple institutions. Metastases have more frequent regional MYC amplification suggesting that MYC is more commonly involved in prostate cancer progression. Although mRNA expression studies clearly indicated MYC overexpression in most human prostate cancer lesions, until recently the phase of prostate cancer development in which MYC protein is expressed in humans remained unclear. It was critical to directly ascertain MYC protein levels given that MYC protein is tightly regulated by posttranscriptional and posttranslational mechanisms

and that the presence of MYC mRNA does not necessarily imply the presence of MYC protein. Several genome-wide association studies have shown that the 8q24 region contains several risk loci that are linked to an increased risk of prostate cancer nevertheless germline variants on MYC expression remain unclear. (Koh et al., 2010).

BIRC5/survivin (chromosome 17) is a member of the inhibitor of apoptosis (IAP) family. The survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. This has been shown by disruption of survivin induction pathways leading to increase in apoptosis and decrease in tumour growth. The survivin protein is expressed highly in most human tumours and fetal tissue, but is completely absent in terminally differentiated cells (Altieri 2010). The molecular mechanisms of survivin regulation are still not well understood, but regulation of survivin seems to be linked to the p53 protein. It also is a direct target gene of the Wnt pathway and is upregulated by beta-catenin (Olie et al., 2000)

It has been observed that the development of hormone resistance in prostate cancer may be due to the upregulation of antiapoptotic genes, one of which is survivin. Therefore if survivin is a significant contributor to the development of hormonal therapy resistance in prostate cancer cells, targeting survivin and blocking it would enhance prostate cancer cell susceptibility to anti-androgen therapy (Zhang et al., 2005).

CDC25 (5q31) is a dual-specificity phosphatase first isolated from the yeast *Schizosaccharomyces pombe* as a cell cycle defective mutant. Cdc25 proteins control entry into and progression through various phases of the cell cycle, including mitosis and S phase. Mammalian cells have three CDC25 proteins, CDC25a, b, and c. The expression patterns of these proteins are mostly overlapping, and the knowledge on the function of each CDC25 protein has been limited. Studies using cultured cells suggest that CDC25a regulates both G1-S and G2-M transitions, whereas CDC25b and CDC25c are involved only in G2-M

regulation (Ray et Kiyokawa 2008). The cell cycle–promoting action of CDC25a and its role as a p53-independent checkpoint target suggest that the expression level of this phosphatase must be tightly controlled in normal cells. Misregulation of CDC25a levels results in impaired checkpoint response to DNA damage with aberrantly high CDK activity. Impaired checkpoint could lead to genomic instability, a hallmark of cancer. Furthermore, CDC25a overexpression often correlates with more aggressive diseases and poor prognosis (Ray et Kiyokawa 2008).

As might be expected for a vital protein, Cdc25 family members are regulated by a complex constellation of at least seven distinct intracellular mechanisms, each of which represents potential small molecule target sites (Lazo et Wipf 2008). Because of the relatively short half-life of them Cdc25 family members (<20 min), one potential target for suppressing the total protein would be inhibition of transcription or translation. There is considerable interest in designing inhibitors of protein-protein interactions and proteasomal activity. Nonetheless, the most obvious small molecule target remains the catalytic site (Lazo et Wipf 2008).

P53 (17p13.1) is a tumor suppressor gene whose mutations are implicated in the molecular genetics of many human malignancies. In fact, p53 is probably the most frequently mutated gene in human cancer. Nucleotide alterations, most commonly single point mutations, have been shown to not only abrogate the p53 suppressor function but also contribute to the transformed phenotype. Mutated p53 alleles typically encode abnormally stable p53 proteins that accumulate to high levels in tumor cell nuclei. Using immunohistochemistry in combination with mutation verification, abnormal nuclear p53 accumulation and p53 mutation have been observed in prostate cancers and the association of mutated alleles with metastasis has been repeatedly detected (Dong et al., 2006). Certain DNA viruses may also contribute to the progression of invasive cancer in infected tissue acting to p53. One of the most effective strategies employed by these viruses is the inhibition of p53 protein by interaction with viral oncoproteins implying a direct but also an

indirect role of these viruses in the impairment of p53 structure and function (Collot-Teixeira et al., 2004). Inactivation of p53 has been proposed as the main mechanism whereby the oncogenic virus BK (BKV) plays a role in early cancer progression as hypothesized by Das and colleagues (Das et al., 2008).

The human polyomavirus BK belongs to the *Polyomaviridae*, a family of small non-enveloped icosahedral DNA viruses which infect human population worldwide. BKV naturally infects humans, it resides in the kidneys in a latent or persistent state reactivating upon immunosuppression and it is usually acquired early in life (90% of adults are seroconverts) (Fioriti et al., 2005). The BK viral genome can be divided into an early region (regulatory proteins), a late region (viral capsid proteins and the agnoprotein) and a regulatory region containing the origin of DNA replication (O-block, 142 base-pairs) and sequences involved in transcriptional regulation of both early and late genes (promoter/enhancer elements), referred to as the transcriptional control region (TCR). The TCR of the proposed archetypal BK strain WW has been arbitrarily divided into four transcription factor binding sequence blocks, called P (68 base-pairs), Q (39 base-pairs), R (63 base-pairs), and S (63 base-pairs) (Fig. 2), (Moens et Johannessen, 2008). These binding sites undergo deletion and enhancement process that could generate variants that could offer advantages to the virus in its host (Sharma et al., 2007).

BKV oncogenic potential is linked especially to large T Antigen. In fact TAg binds and blocks p53. Nevertheless another viral protein named small t antigen, has oncogenic properties. Its function in cellular transformation is still under study but it probably cooperates with TAg in inducing S phase. Finally, an interesting role in cancer development could be played by TCR. In no permissive cells TCR undergoes to rearrangement process and viral variants with a transformation potential could be selected. In particular binding sites for oncoproteins or tumor suppressors could be selected to promote cell proliferation (Fig.3) (Fioriti et al., 2005).

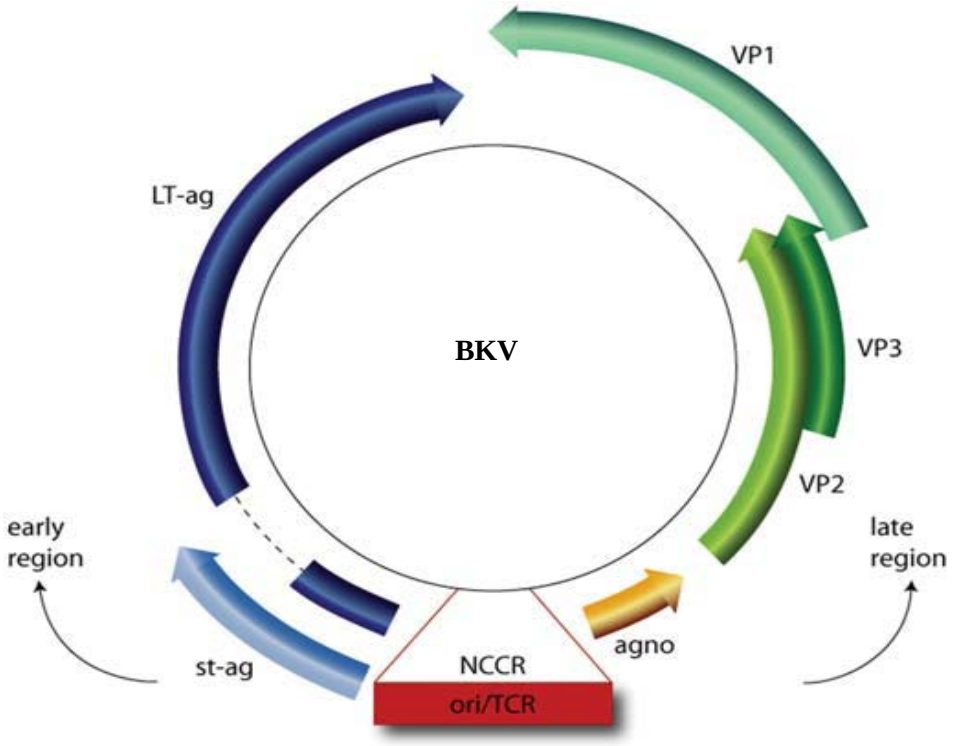

Figure 2: Schematic representation of the circular double stranded DNA genome of the Human Polyomavirus BK. The early region: LT-ag (Large T Antigen), st-ag (small t antigen). The late region: agnoprotein, capsid proteins (VP1, VP2 and VP3). The transcription control region (NCCR/TCR).
Moens et Johannessen . J Dtsch Dermatol Ges. 2008;6(9):704-708.

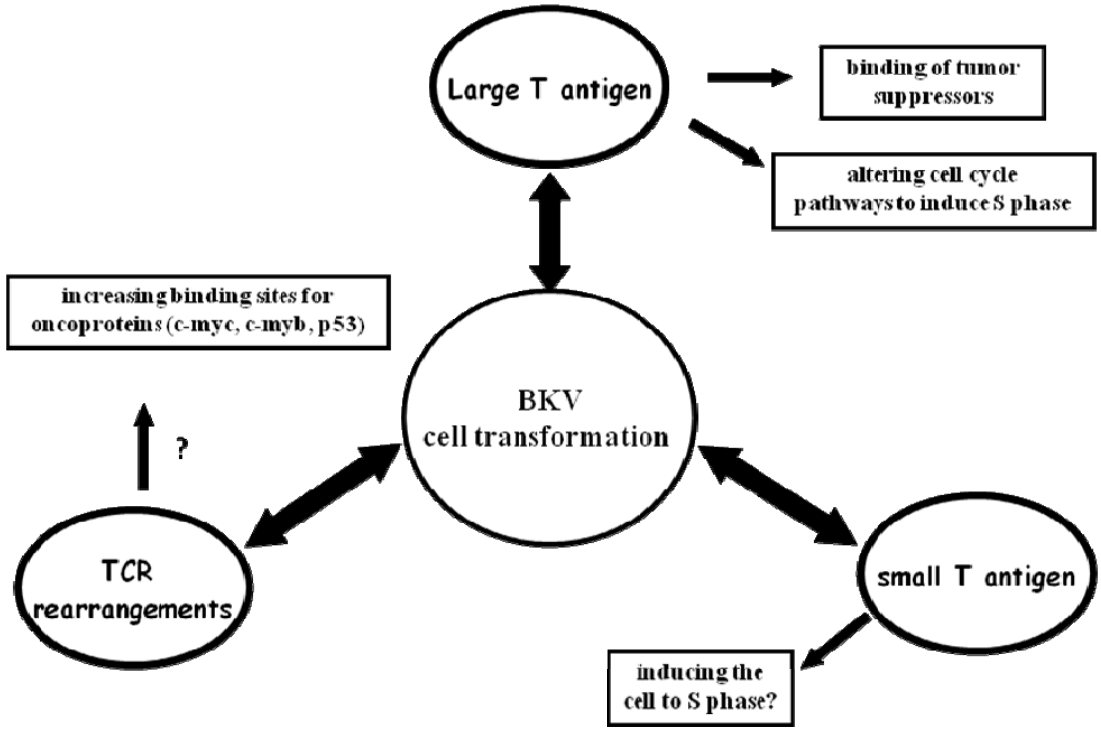

Figure 3: Schematic representation of the oncogenic properties of the Human Polyomavirus BK.

PROPOSALS

Taking in account the molecular and the oncoviral portrait, during these years, the research was aimed to understand if normal prostate cells are really susceptible to viral infection and if BKV could play a role in transformation scenario. In addition, p53 localization into infected prostate cancer cells was investigated, its mutational analyses was performed and finally expression pattern of selected PC genes were studied.

MATERIALS AND METHODS

Patients population

Tumor samples were selected on the basis of parameters accounting for tumor heterogeneity such as histopathological diagnosis, tumor size, grade and stage, the androgen receptor status and family history data.

The samples examined (urine, blood, fresh and paraffin embedded tissue samples) were from patients admitted to the ‘‘Umberto I’’ Hospital of Rome, Italy and without a history of neoplastic diseases. For each patient, informed consent, clinical-pathological and follow-up data are available in a computerized registry database.

In the first two years, 26 patients (median age of 66) with prostate adenocarcinoma clinically proven pT2aN0M0 or pT3N0M0 were analysed. As controls 12 patients (median age of 69) with histological diagnosis of BHP were examined.

In the third year of the study, fresh biopsies of 15 patients (median age of 60) with clinically pT3N0M0 PC were analysed. As controls, non tumour biopsies of the same patients were analyzed.

Finally in last year, 30 fresh biopsies (64 mean age, pT3N0M0 adenocarcinoma) were analysed using as controls non tumour biopsies of the same patients.

The patients were enrolled in this study according to the following criteria:

(1) no previous hormonal or radiation therapy;

(2) no previous surgery on the prostate gland;

(3) histologically proven prostate cancer by biopsy and confirmed by radical prostatectomy;

BKV sequences were searched in urine, blood and fresh PC samples by means of quantitative assay. In addition, BKV TAg and tumor suppressor p53 localization in neoplastic cells was examined by immunohistochemistry (IHC) in paraffin-embedded PC samples with antibodies specific to TAg or p53. The sequencing analysis of p53 specific DNA binding exons (5–9) was carried out to understand if p53 mutations might be correlated with viral infection and/or cancer progression.

Successively, since specific antibody for BKV was out of the market, IHC was not carried out. Nevertheless, other target genes (C-MYC, BIRC5/SURVIVIN, CDC25) than P53 , in PC gene profile, were considered. Genes were chosen using ONCOMINE database and scientific literature. Their expression was investigated using Retro Transcriptional Quantitative PCR (RT-QPCR) whereas BKV DNA and TAg RNA were searched using Quantitative PCR (QPCR) and RT-QPCR.

Finally in last year, target genes and viral sequences were searched using the same techniques mentioned above. In addition Transcriptional Control Region of BKV was searched by nested PCR and when it was found, it was sequenced (Fioriti et al., 2005).

Clinical Specimens Processing

Urine. One milliliter of urine sample, collected without preservatives, was incubated in lysis buffer and proteinase K (200 mg/ml). DNA extraction was performed by the DNeasy® Tissue Kit (QIAGEN, S.p.A, Italy) according to the

manufacturer's instructions. One microgram of total purified DNA was used for Q-PCR. Blood.

Blood. Blood samples, collected in 4-mL Vacuntainer® tubes containing EDTA (BD Becton Dickinson S.p.A, Italia), were centrifuged at 1,376 g/sec for 10 min and 200 μl of plasma were incubated in lysis buffer and proteinase K (200 mg/ml). DNA extraction was performed by the QIAmp® DNA Blood Kit (QIAGEN) according to the manufacturer's instructions. One microgram of purified DNA was used for Q-PCR.

Biopsies. Fresh and paraffin-embedded prostate cancer resections were obtained from each patient. In parallel, as controls, fresh, and paraffin-embedded benign prostatic hyperplasia resections were analyzed. About 25 mg of each fresh sample were incubated in lysis buffer and proteinase K (200 mg/ml). DNA extraction was performed by the DNeasy® Tissue Kit (QIAGEN) according to the manufacturer's instructions. RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was obtained using High Capacity RNA-to-cDNA Products, (Invitrogen) following manufacturer's manual. One microgram of total purified DNA was used for Q-PCR whereas two micrograms of total purified cDNA were used for RT-QPCR. Immunohistochemistry was carried out using about 25 mg of paraffin-embedded prostate cancer and benign prostatic hyperplasia resections. Samples were deparaffinized, rehydrated, and subjected to high temperature antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by 3% H_2O_2.

Immunohistochemistry

Immunohistochemical testing was performed on paraffin embedded sections using specific antibodies against BKV-TAg and p53 proteins. The primary antibodies used were the mouse monoclonal antibody for BKV Large TAg

(PAb416, 1:200 dilution in blocking buffer, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) and the mouse monoclonal antibody for p53 protein (p53Ab-6, Clone DO-1; 1:20 dilution in blocking buffer, Lab Vision, Fremont, CA). The incubation was carried out for 1 hr at room temperature; the secondary antibody used for both TAg and p53 proteins was biotinylated goat anti-mouse IgG. All slides were processed using preformed horseradish peroxidaseconjugated streptavidin (Biogenex, San Ramon, CA). The immunoreaction product was revealed using aminoethylcarbazole (AEC) and 0.01% H_2O_2 (Biogenex, San Ramon, CA). Negative control staining was carried out by omitting the primary antibody. Sections were counterstained in Mayer's acid hemalum and analyzed.

Quantitative PCR for BKV DNA and viral TAg cDNA

Urine, blood, fresh prostate cancer and benign prostatic hyperplasia specimens were tested using Q-PCR for detection and quantitation of BKV-DNA. Q-PCR assay was performed using 7300 Real Time PCR System (AB Applied Biosystems, Foster City, CA). PCR amplifications were run in a reaction volume of 20 µl containing 5 µl of DNA sample, Q-Amplimaster (reaction mix for quantitative amplification), Q-Amplimix (forward and reverse primers), and finally Q-Ampliprobe (hydrolysis probes) (Nanogen Advanced Diagnostics, S.r.l., San Diego, CA). Thermal cycling was initiated with a first denaturation step of 10 min at 95°C, followed by 45 cycles of 95°C for 15 sec, 60°C for 1 min, and 72°C for 1 min, at the end of which fluorescence was read. The amplification data were analyzed with software provided by the manufacturer. Standard curves for the quantitation of BKV were constructed using serial dilutions of a plasmid containing target sequences for BKV (TAg). The plasmid concentrations ranged from 10^2 to 10^5 plasmid copies of BKV-DNA target. All patient samples were tested in triplicate and the number of BKV copies in each sample was calculated from the standard curve. Q-PCR results for urine and plasma specimens were expressed as copies of viral DNA per milliliter (c/ml) of sample and as copies per

10^5 cells (c/10^5 cells) of sample for the biopsies. To correct for the variable amount of DNA in different tissue samples, each sample was subjected to simultaneous TaqMan PCR for the housekeeping gene Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, Accession No. J04038), targeting the region between exons 6 and 8. Results were considered acceptable only in the presence of GAPDH-positivity (Costa et al. 2009). Standard precautions designed to prevent contamination during Q-PCR were followed. A not template control (NTC) lane was included in each run. The β-globin gene was used as internal standard (Nanogen Advanced Diagnostics, S.r.l., San Diego, CA). Q-PCR allows the detection of 10 molecules of target sequences in 5 µl of extracted DNA used in the reaction.

Quantitative PCR for C-MYC, BIRC5/SURVIVIN and CDC25

All reagents were spinned for 10-15 seconds, then, in a PCR tube, the following reagents were added: 12.5 µl of RT² SYBR Green qPCR Master Mix, 1.5 µl of high-quality, nuclease-free H_2O, 10 µl of cDNA (template) and 1.0 µl of gene-specific RT² qPCR specific primers. Tubes were quickly centrifuged and placed in a 7300 Real Time PCR System (AB Applied Biosystems, Foster City, CA). Two-step cycling program was used, thermal cycling was initiated with a first denaturation step of 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. SYBR Green fluorescence from every well was detected and recorded during the annealing step of each cycle. Specific gene expression was quantified by using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Normalization of gene expression was performed using GAPDH as reference gene.

PCR for BKV TCR and amplicons sequencing

Nested PCR employed two pairs of primers that anneal to the invariant regions flanking the TCR of BKV. Primers BKTT1 (+) and BKTT2 (-) generated a 748 bp DNA fragment. The second pair, BK1 (+) and BK2 (-), amplified a portion of the

first round PCR product, generating a fragment of 354 bp. The PCR precautions and conditions followed are described in Fioriti and co-workers paper (Fiorit et al., 2005). PCR products corresponding to BKV TCR were purified and sequenced. Briefly, amplified products were purified prior sequencing to remove the excess of primers with QIAquick® PCR purification kit, according to QIAGEN protocol. DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, mod. 370 A), according to manufacturer's specifications (Amplicycle Kit, Applied Biosystem). Sequences were organised and analysed using the Genetic Computer Group sequence analysis software package.

p53 Mutational Analysis

The mutational analysis of p53 was performed by SNPCapture™p53 Mutation Screening Kit (Panomics, Fremont, CA) and sequence analysis (Das et al., 2008). SNPCapture™p53 Mutation Screening Kit (Panomics) is a gel-based method designed to detect genetic mutations in coding regions between exons 5 and 9 of the human gene p53 through Holliday junction formation. The reason for this restriction is that more than 98% of p53 mutations in human neoplasias are located in these exons. In addition, exons 5–9 (codons 126 and 331 with 540 base pairs) contain DNA sequences that code for domains necessary for sequence-specific DNA binding. This assay includes primers for sequence-specific detection, convenient premixed reagents, control wild-type, and mutant p53 templates. Control wild-type templates were designed on the basis of GenBank data. Mutant templates contain missense mutations localized in the following codons: 175 (exon 5), 213 (exon 6), 249 (exon 7), and 273 (exons 8 and 9). Extracted DNA from biopsies (target DNA) and the p53 wild-type DNA,which functions as a reference, were amplified in a thermal cycler. Amplification was performed by a preliminary Taq polymerase activation step at 95°C for 10 min, followed by 45 cycles of 15 sec at 94°C for DNA denaturation, 23 sec at 58°C for primers annealing, and 1 min at 72°C for DNA extension. Five microliters of target DNA(approximately 5 ng) was

used in 50 ml of PCR reaction. Amplified target DNA was mixed with amplified reference DNA (p53 wild-type DNA) and subjected to a 65°C temperature for 30 min to form stable Holliday junction between mutated targets and reference DNA. Holliday junctions were detected by electrophoresis on 1% agarose gel stained with ethidium bromide. All assays were carried out in a GeneAmp® PCR System 9700 (AB Applied Biosystems). Although the use of SNPCapture™p53 Mutation Screening Kit not required a confirming sequencing analysis, a p53 sequencing analysis was carried out according to Das et al. in order to detect other point mutations not revealed by the commercial kit. After gene amplification, PCR products were separated by agarose gel electrophoresis, extracted (Qiaquick gel extraction kit; QIAGEN S.p.A) and sequenced by automatic DNA sequencer (Applied Biosystem, mod. 370A), according to manufacturer's specifications (Amplicycle Kit, Applied Biosystem). Sequences were organized and analyzed using the Genetic Computer Group sequence analysis software package.

Statistical analysis

Data were summarized as medians and ranges or as mean, as appropriate. If Z test indicated a non-normal distribution, we used nonparametric test such as Mann–Whitney U test and Kruskal–Wallis test. Categorical data were analyzed by using χ^2 test and Student's t test. P values < 0.05 were considered statistically significant.

RESULTS AND CONCLUSIONS

In the first two years, the analysis of urine, blood and fresh tissue samples taken from 26 patients with prostate cancer, revealed the presence of BKV in the urine of 14/26 (54%) patients, whereas 8/26 (31%) blood samples were positive for viral DNA. No control subject was found positive (data not shown). Biopsies examination evidenced BKV DNA in 22/26 (85%) (Table 3). The average of viral copies number was calculated on biopsies of positive patients belonging to the same Gleason score class. Seven patients with Gleason 9 showed an average of 16,914 c/10^5 cells, whereas in six patients with Gleason 8 an average of 13,300 c/10^5 cells was found and finally seven patients with Gleason 7 showed an average of 9,457 c/10^5 cells (Table 4). Regarding patients with Gleason 6 score, two patients resulted positive to BKV DNA with a low mean copy number of 1,000 c/10^5 cells (data not shown). Negative patients had Gleason 7 score (one patient) and Gleason 6 score (three patients) (data not shown). No control subject was found positive (data not shown).

In the third year 15 biopsies of 15 patients were examined and BKV DNA was found in 3/15 (20%) biopsies (Table 3). In particular, 2 positive specimens belonging to patients with Gleason score 9 showed an average of 15,490 c/10^5 cells whereas, in the remaining positive sample taken from a patient with Gleason score 7, 8352 viral copies were found (Table 4). About controls, BKV was detected only in one (data not shown).

In the last year 30 patients were analysed. Results showed that 6 out of 30 patients (20%) were BKV infected whereas in the remaining 24 patients BKV DNA was absent (Table 3). In particular the BKV DNA average detected in 3 patients belonging to Gleason score 9 was of 15,333 c/10^5 cells, whereas an average of 11,761 viral copies was calculated for the 2 samples taken from the patients with Gleason score 8. Finally in the one positive specimen obtained from a

patient with Gleason score 7, 7,800 BKV DNA copies were detected (Table 4). No control was found positive (data not shown).

Regarding statistical analysis, the comparison between the mean values of BKV DNA copies number obtained for each class of Gleason scores over the 4 years of research was not statistically significant, although it is possible to observe a decrease of copies number from the class with highest Gleason score (9) to that with lower Gleason score (7)

Table 3: Specimens positive for the presence of BKV DNA

YEAR OF STUDY	POSITIVE SAMPLES FOR BKV					
	URINE		BLOOD		PC SPECIMENS	
	Pos	Neg	Pos	Neg	Pos	Neg
1-2	14	12	8	18	22	4
3	Not received		Not received		3	12
4	Not received		Not received		6	24

Table 4: Mean quantity of BKV DNA copies number found in patients with different Gleason score

GLEASON SCORE	BIOPSIES			
	YEAR 1-2	YEAR 3	YEAR 4	P*
	7 positive specimens	2 positive specimens	3 positive specimens	
9	16,914 copies/10^5 cells	15,490 copies/10^5 cells	15,333 copies/10^5 cells	0.20
GLEASON SCORE	6 positive specimens		2 positive specimens	
8	13,300 copies/10^5 cells		11,761 copies/10^5 cells	0.20
GLEASON SCORE	7 positive specimens	1 positive specimen	1 positive specimen	
7	9,457 copies/10^5 cells	8,352 copies/10^5 cells	7,800 copies/10^5 cells	0.10

***by Mann-Whitney U test**

Immunohistochemistry was performed only on specimens analysed during the first two years because of specific antibody for BKV then was out of the market. Results showed the presence of BKV-TAg protein on scattered cells of the prostate cancer glandular epithelium (Fig. 4A), whereas in benign prostatic hyperplasia, BKV-TAg was not detected (Fig. 4B). p53 and TAg localization was observed in the cytoplasm of the neoplastic glandular epithelial cells (Fig. 5A), whereas in the TAg negative tumors, p53 was localized in the nucleus (Fig. 5B). Immunohistochemistry carried out on control specimens showed low levels of p53 into the glandular epithelial cells' cytoplasm (data not shown).

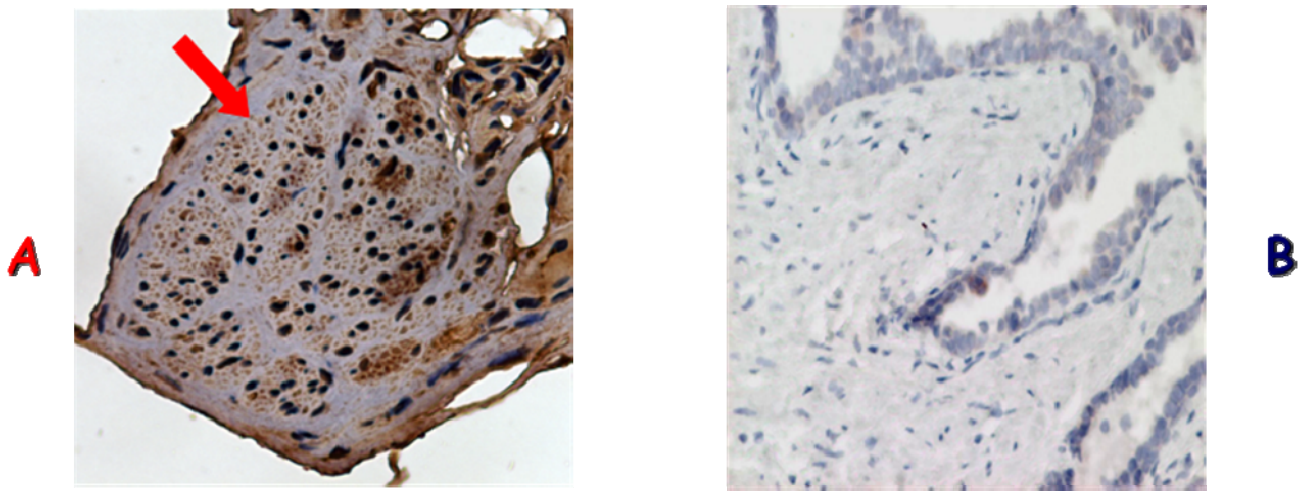

Figure 4A: Expression of TAg in prostate sample immunostained with anti-TAg Ab. Tumoral prostate section derived from a BKV infected patient corresponding to sample 5. The section shows cuboidal epithelium cells labeled with anti-polyomavirus (BKV) (red arrow).

Figure 4B: The section shows a BHP section derived from a negative subject for BKV infection.

Original magnification 20X.

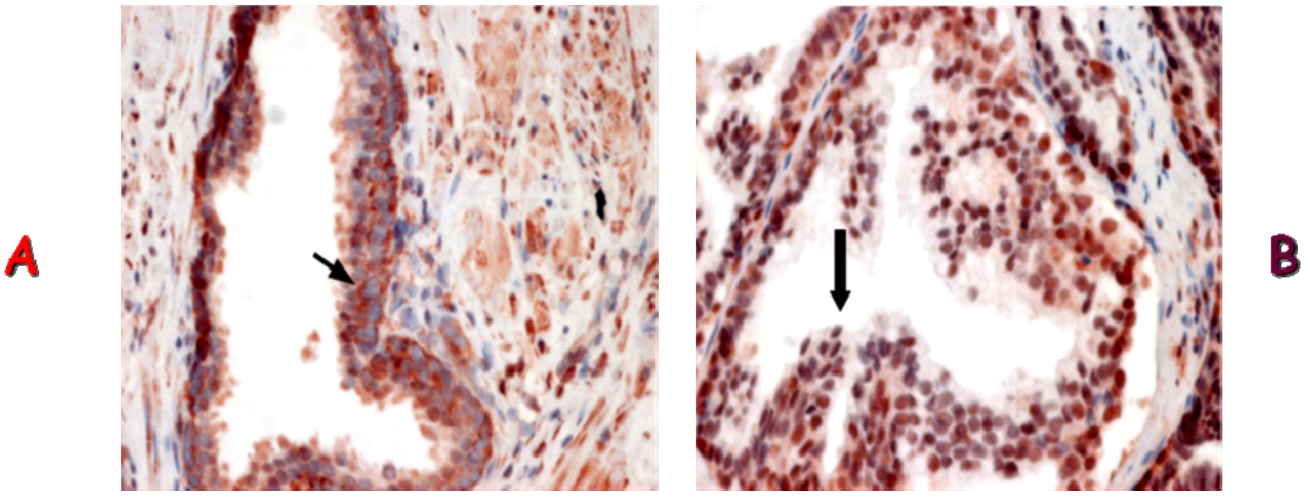

Figure 5A. Immunohistochemical analysis of p53 expression on prostate cancer sections from BKV-infected and uninfected patients. Prostate section from BKV-infected patient (sample 5) showing p53 expression. Positivity is present in the cytoplasm of cuboidal epithelium cells (black arrow).

Figure 5B: Prostate section from uninfected patient (sample 25) showing p53 expression. Strong positivity is present in the nucleus of cuboidal epithelium cells (black arrow).

Original magnification 20X

Regarding p53 mutational analysis, all specimens analysed during the four years of the study, had at least one mutated exon of p53. It was found that codons 248 (exon 7) and 273 (exon 8) were more susceptible to mutation for all examined patients (Dong, 2006; Web-Site: IARC TP53 Mutation Database, 2006). Exon 7 was always mutated in patients with Gleason grade 9. Mutations in exons 5, 6, 8 and 9 were observed in 12/22 positive specimens analysed during the first two years, patients; regarding the 3 positive BKV patients examined during the third year, 2/3 patients had mutation in exons 6 and 8, 1/3 patient in exons 5, 8 and 9. Finally, exons 5 and 6 were found mutated in 4/6 BKV positive patients studied in the four year whereas the remaining 2 patients had mutation in exon 8. Results are shown in figure 6 and table 5.

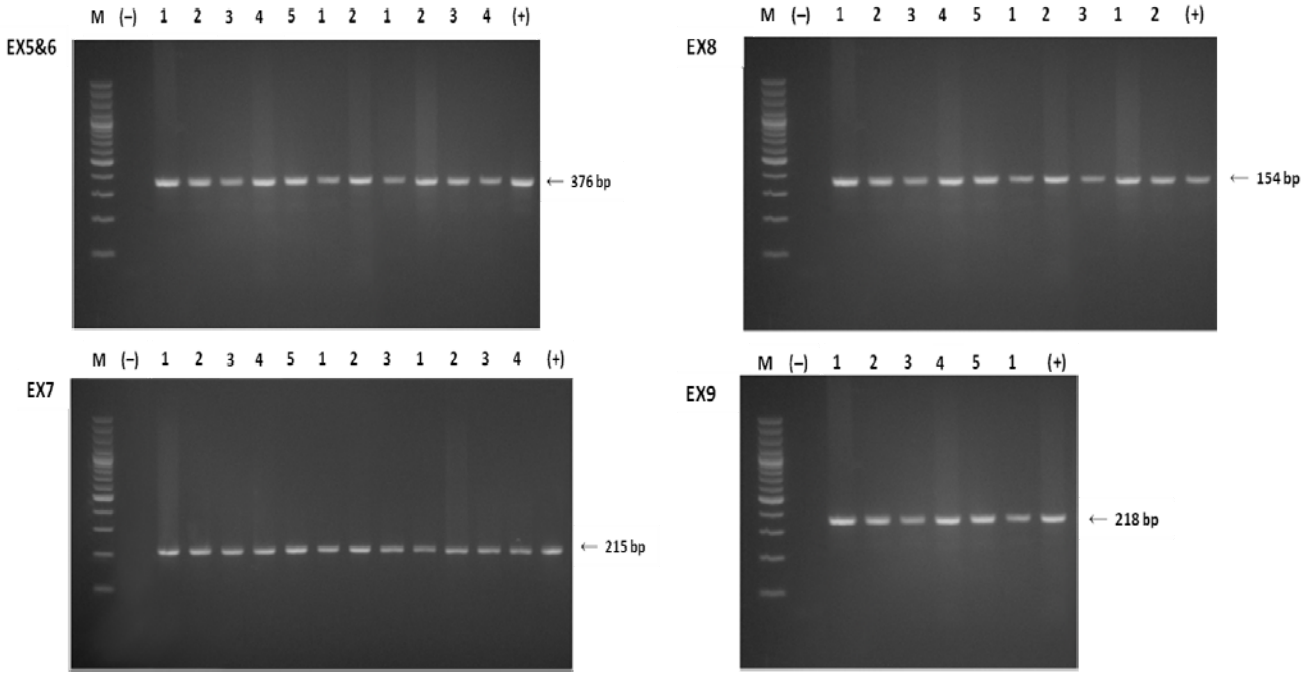

Figure 6: p53 gene amplification. PCR products are visualized by electrophoresis on ethidium bromide-stained 2% agarose gel in 1xTBE buffer. M: low molecular weight marker DNA (100 bp MBI Fermentas);

Ex 5&6 (–): negative control; lanes 1, 2, 3, 4, 5: BKV positive PC samples of first two years, lanes 1, 2: BKV positive PC samples of third year, lanes 1, 2, 3, 4: BKV positive PC samples of fourth year (+): positive control.

Ex 7 (–): negative control; lanes 1, 2, 3, 4, 5: BKV positive PC samples of first two years, lanes 1, 2: 3, BKV positive PC samples of third year, lanes 1, 2, 3, 4: BKV positive PC samples of fourth year (+): positive control.

Ex 8 (–): negative control; lanes 1, 2, 3, 4, 5: BKV positive PC samples of first two years, lanes 1, 2: 3, BKV positive PC samples of third year, lanes 1, 2,: BKV positive PC samples of fourth year (+): positive control.

Ex 9 (–): negative control; lanes 1, 2, 3, 4, 5: BKV positive PC samples of first two years, lanes 1: BKV positive PC samples of third year, lanes 1, (+): positive control.

Table 5: An example of multiple sequence alignment for p53 gene (exon 5).

```
                                  130
P53 ex.5          TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
                            Patients of the first two years
Pz.1              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.2              TACTCCCCTGCCTTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.3              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.4              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.5              TACTCCCCTGCCTTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
                             Patients of the third year
Pz.1              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
                            Patients of the fourth year
Pz.1              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.2              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.3              TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
Pz.4              TACTCCCCTGCCTTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTG 60
                  ************ ********************************************

P53 ex.5          TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
                            Patients of the first two years
Pz.1              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.2              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.3              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.4              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.5              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
                             Patients of the third year
Pz.1              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
                            Patients of the fourth year
Pz.1              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.2              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.3              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
Pz.4              TGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAG 120
                  ************************************************************
                                        175
P53 ex.5          TCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
                            Patients of the first two years
Pz.1              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.2              TCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.3              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.4              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.5              TCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
                             Patients of the third year
Pz.1              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
                            Patients of the fourth year
Pz.1              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.2              TCACAGCACATGACGGAGGTTGTGAGGCACTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.3              TCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
Pz.4              TCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGC 180
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```
P53 ex.5          GATG 184
Patients of the first two years
Pz.1              GATG 184
Pz.2              GATG 184
Pz.3              GATG 184
Pz.4              GATG 184
Pz.5              GATG 184
Patients of the third year
Pz.1              GATG 184
Patients of the four year
Pz.1              GATG 184
Pz.2              GATG 184
Pz.3              GATG 184
Pz.4              GATG 184
                  ****
```

Table 5: An example of multiple sequence alignment for p53 gene (exon 6).

```
                                  191
P53 ex.6          GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
                           Patients of the first two years
Pz.1              GTCTGGCCCCTCTTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.2              GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.3              GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.4              GTCTGGCCCCTCTTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.5              GTCTGGCCCCTCTTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
                             Patients of the third year
Pz.1              GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.2              GTCTGGCCCCTCTTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
                            Patients of the fourth year
Pz.1              GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.2              GTCTGGCCCCTCGTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.3              GTCTGGCCCCTCTTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
Pz.4              GTCTGGCCCCTCGTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG 60
                  ************ ********************************************
                                                214
P53 ex.6          ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
                           Patients of the first two years
Pz.1              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.2              ATGACAGAAACACTTTTCGACGTAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.3              ATGACAGAAACACTTTTCGACGTAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.4              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.5              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
                             Patients of the third year
Pz.1              ATGACAGAAACACTTTTCGACGTAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.2              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
                            Patients of the fourth year
Pz.1              ATGACAGAAACACTTTTCGACGTAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.2              ATGACAGAAACACTTTTCGACGTAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.3              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
Pz.4              ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAG 113
                  ********************* ******************************
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Table 5: An example of multiple sequence alignment for p53 gene (exon 7)

```
P53 ex.7        GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
                         Patients of the first two years
Pz.1            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.2            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.3            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.4            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.5            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
                           Patients of the third year
Pz.1            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.2            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.3            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
                           Patients of the fourth year
Pz.1            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.2            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.3            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
Pz.4            GTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC 60
                ************************************************************
                          248       251
P53 ex.7        GGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
                         Patients of the first two years
Pz.1            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.2            GGCATGAACCGGAGGCCCAGCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.3            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.4            GGCATGAACTGGAGGCCCAGCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.5            GGCATGAACCGGAGGCCCAGCCTCACCATCATCACACTGGAAGACTCCAG 110
                           Patients of the third year
Pz.1            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.2            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.3            GGCATGAACCGGAGGCCCAGCCTCACCATCATCACACTGGAAGACTCCAG 110
                           Patients of the fourth year
Pz.1            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.2            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.3            GGCATGAACTGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAG 110
Pz.4            GGCATGAACCGGAGGCCCAGCCTCACCATCATCACACTGGAAGACTCCAG 110
                ********* ********* ******************************
```

Table 5: An example of multiple sequence alignment for p53 gene (exon 8)

```
                                        267                 273
P53 ex.8        TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGA 60
                         Patients of the first two years
Pz.1            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGTGTGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.2            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGTGTGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.3            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.4            TGGTAATCTACTGGGATGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.5            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGGAGAGA 60
                          Patients of the third year
Pz.1            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.2            TGGTAATCTACTGGGATGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.3            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGTGTGTTTGTGCCTGTCCTGGGAGAGA 60
                           Patients of the fourth year
Pz.1            TGGTAATCTACTGGGACGGAACAGCTTTGAGGTGCGAGTTTGTGCCTGTCCTGGGAGAGA 60
Pz.2            TGGTAATCTACTGGGATGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGAGA 60
                ********** ********** ********** **********

P53 ex.8        CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
                         Patients of the first two years
Pz.1            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.2            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.3            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.4            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.5            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
                          Patients of the third year
Pz.1            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.2            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.3            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
                           Patients of the fourth year
Pz.1            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
Pz.2            CCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGAGCTGCCCCC 120
                ************************************************************

TP53 ex.8       AGGGAGCACTAAGCGAG 137
Patients of the first two years
Pz.1            AGGGAGCACTAAGCGAG 137
Pz.2            AGGGAGCACTAAGCGAG 137
Pz.3            AGGGAGCACTAAGCGAG 137
Pz.4            AGGGAGCACTAAGCGAG 137
Pz.5            AGGGAGCACTAAGCGAG 137
Patients of the third year
Pz.1            AGGGAGCACTAAGCGAG 137
Pz.2            AGGGAGCACTAAGCGAG 137
Pz.3            AGGGAGCACTAAGCGAG 137
Patients of the fourth year
Pz.1            AGGGAGCACTAAGCGAG 137
Pz.2            AGGGAGCACTAAGCGAG 137
                *****************
```

Table 5: An example of multiple sequence alignment for p53 gene (exon 9)

```
P53 ex.9          CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
                           Patients of the first two years
Pz.1              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
Pz.2              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
Pz.3              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
Pz.4              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
Pz.5              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
                             Patients of the third year
Pz.1              CACTGCCCAACAACACCAGCTCCTCTCCCCAGCCAAAGAAGAAACCACTGGATGGAGAA 59
                  ************************************************************
                  327
P53 ex.9          TATTTCACCCTTCAG 74
Patients of the first two years
Pz.1              TAGTTCACCCTTCAG 74
Pz.2              TAGTTCACCCTTCAG 74
Pz.3              TAGTTCACCCTTCAG 74
Pz.4              TAGTTCACCCTTCAG 74
Pz.5              TAGTTCACCCTTCAG 74
Patients of the third year
Pz.1              TAGTTCACCCTTCAG 74
                  * ************
```

Overall the results lead to the hypothesis that the presence of BKV genome and, more importantly, the expression of its TAg early protein, could suggest that this virus may play a role in the development or progression of prostate cancer. In particular BKV TAg might contribute to cellular transformation process triggered by p53 gene mutations in the region encoding for DNA binding site domains. These results might suggest that, in heterozygote subjects for p53 gene mutations, the poor quantity of wild type p53 protein could be sequestered from viral AgT promoting the development of a neoplastic phenotype.

From the third year, in order to understand if prostate cancer cells present a particular genotype, target genes C-MYC, BIRC5/SURVIVIN, CDC25 than P53 were considered. Genes were chosen using ONCOMINE database and scientific literature. Their expression was investigated using RT-QPCR. According to scientific production, results showed the upregulation of C-MYC at the mRNA level (Fig.7). In addition, since in normal cells c-Myc RNA has a short half live (about 30 min), in cancer cells the gene expression is uncontrolled and MYC amplification could be related to PC progression or metastatization, so,

understanding the role of c-Myc in PC is important to yield insights that might be of therapeutic importance.

Also Survivin expression was enhanced in samples analysed (Fig.7). Survivin can be regarded as an oncogene as its aberrant overexpression in most cancer cells contributes to a phenotype more resistant to apoptotic stimuli and chemotherapeutic therapies allowing continued proliferation and survival. P53's normal function is to regulate genes that control apoptosis. As survivin is a known inhibitor of apoptosis, it can be implied that p53 repression of survivin is one mechanism by which cells can undergo apoptosis upon induction by apoptotic stimuli or signals (Mirza et al., 2002). When loss of wild-type p53 occurs, survivin is over-expressed in the cells contributing to cancer progression.

Regarding CDC25 gene, its overexpression in cancer cells, reflects data literature that evidence its role as a protoncogene (Fig.7). The Cdc25 phosphatases (a,b,c) function as key regulators responsible of the cell cycle during normal eukaryotic cell division and as mediators of the checkpoint response in cells Cdk/cyclin complexes. Their role in cancer onset is more complicated than that of a simple driver of cell proliferation. It is possible to hypothesize that Cdc25 overexpression in tumors is required to circumvent the checkpoints that would otherwise hinder cell proliferation.

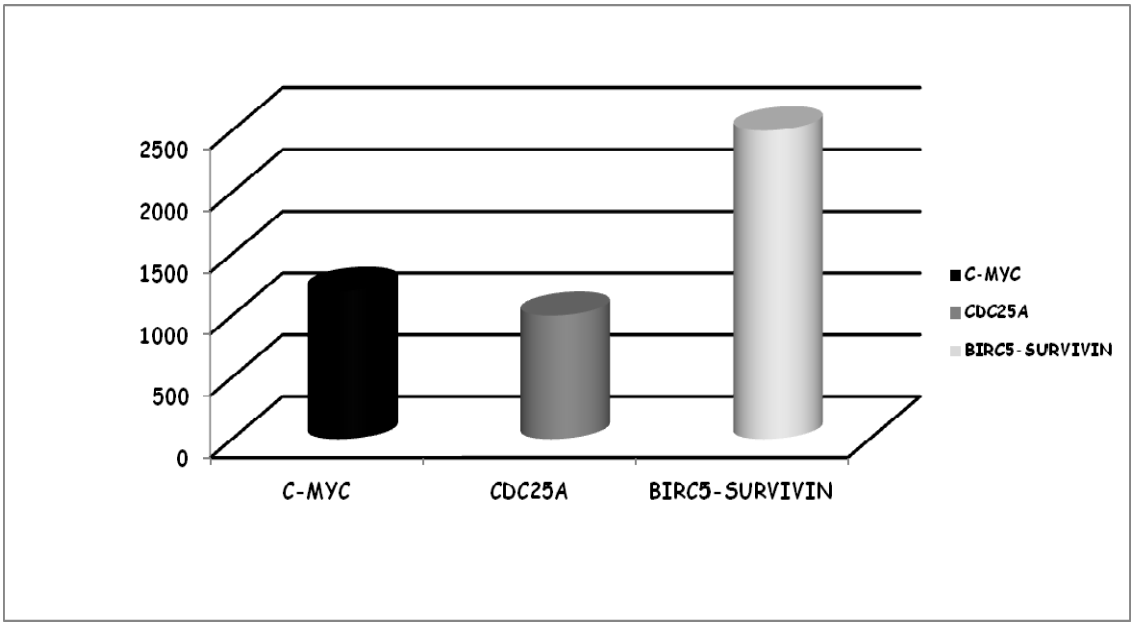

Figure 7: Expression of target PC genes in all samples analysed during the third and fourth year of the study (c/10^5 cells).

To understand if prostate cells are really non permissive cells for BKV replication, searching of viral messengers were perfomed. In particular, since the early protein Large T antigen is necessary to support viral DNA replication, its mRNA was searched. Data revealed that messengers amounts were so low to be incompatible with a real replication (data not shown). Nevertheless, taking in account results obtained during the first two years of the study, it is not possible to exclude that low levels of messengers could be translated in low copies of protein able to interfere with control of cell replication conferring the infected cells properties characteristic of cancer, such as, loss of growth control. In fact, in non permissive cells, BKV activates the cellular DNA replication machinery and other genes involved in S phase progression, but, virions are not produced. This aberrant stimulation of the cell cycle could contribute to oncogenic transformation. Moreover, TAg, preparing the cellular metabolism to support optimal viral replication, deregulates cell's control cycle pathways inducing cell proliferation. Nevertheless it is clear that, in their natural hosts, tumor induction derives from a combination of circumstances and is not a part of the normal virus life cycle.

Finally, an interesting role in cancer development could be played by TCR rearrangements. In this study, two TCR variants were found in samples taken from patients of the fourth year of the study (Fig. 8 and Fig.9). These variants were characterized by enhancement of c-myc and p53 binding sites. In no permissive cells, binding sites for oncoproteins or tumor suppressors could be selected to promote cell proliferation so rearrangements could enhance transformation potential of the virus. For example, c-myc could be bind by virus to prevent its accumulation into the cell that, otherwise, would be eliminated through protective apoptosis. Alternatively, p53 could be sequestered either by TAg either by viral TCR to block its role as the "guardian of the genome". Any case, TCR rearrangements probably represent adaptive changes conferring increased "viral fitness" in host-cell environment.

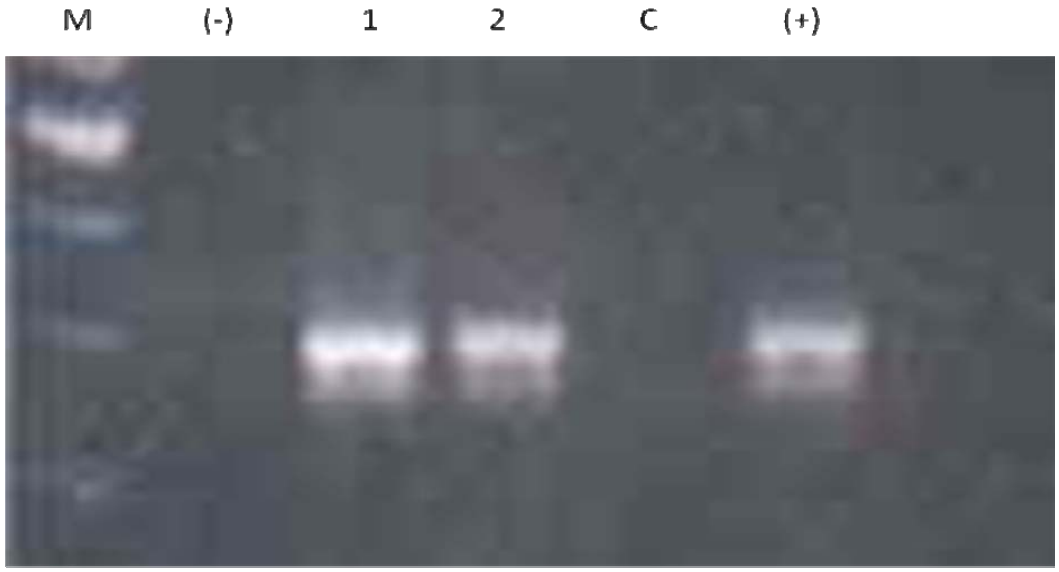

Figure 8: Transcriptional Control region of BKV amplification. PCR products are visualized by electrophoresis on ethidium bromide-stained 2% agarose gel in 1xTBE buffer. M: low molecular weight marker DNA (100 bp MBI Fermentas);

(-) negative control; lanes 1, 2: BKV positive PC samples of the fourth year, lane C: Non tumour biopsy; (+): positive control.

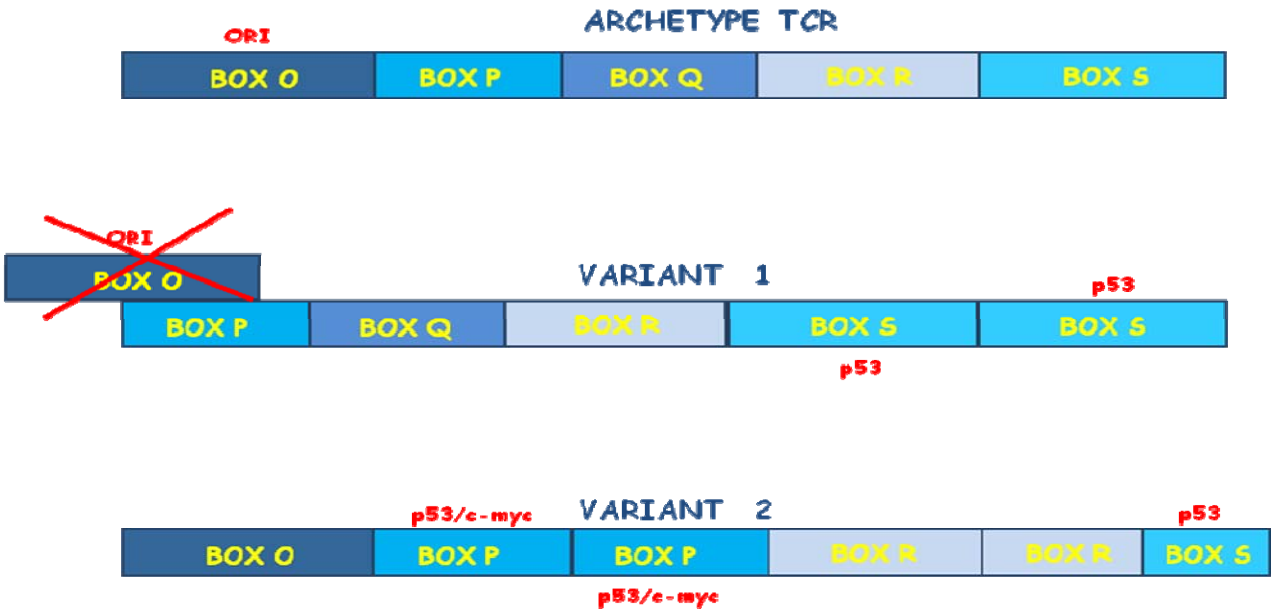

Figure 9: Schematic representation of BKV TCR variants found in two samples taken from patients of the fourth year of the study.

In conclusion, data underline that specific target genes could be a molecular marker for early detection of certain cancers. In particular understudying how their expression change on changes of their regulators (i.e. the signal transducers and activators of transcription) it is of a great importance for cancer drug discovery and therapies. About BKV, DNA presence doesn't exclude viral pressure on cell transformation. In particular the presence of TCR variants allows to hypothesize that they could vary cell expression profile aiding immortalization. However, more studies are necessary to ascertain how to use genes pattern expression for cancer therapy and how BKV could operate on PC susceptibility.

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