

## Emerging immunotherapeutic strategies targeting telomerases in genitourinary tumors

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### Highlights

- Telomerase activity is essential for the pathogenesis of genitourinary tumors.
- Telomerase degraded fragments on tumor cells allow the recognition by immune cells.
- Active and passive immunotherapies are under evaluation in genitourinary tumors.

### Abstract

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Telomerase activity and telomere length are essential for the pathogenesis of several human diseases, including genitourinary tumors. Telomerase constitutes a complex system that includes human telomerase reverse transcriptase (hTERT), human telomerase RNA component (hTR) and telomerase associated protein 1 (TEP1), which are overexpressed in tumor cells compared to normal cells and are involved in the carcinogenesis and progression of renal cell carcinoma (RCC), bladder (BC) and prostate cancer (PCa). In addition, telomerase degraded peptide fragments expressed on the surface of tumor cells lead to their recognition by immune cells. On this scenario, in vitro and in vivo studies have shown effective anti-tumor activity of hTERT-tailored strategies in genitourinary tumors, including active immunotherapy with hTERT-peptide vaccines and passive immunotherapy with hTERT-transduced T cell infusion. This review emphasizes the role of telomerase in the carcinogenesis and progression of genitourinary tumors, thus underlying the potential of emerging telomerase-tailored immunotherapies in these patients.

**Keywords:** Bladder cancer; Immunotherapy; Prostate cancer; Renal cancer; Telomerases.

## 1. Introduction

In the last decade, immunotherapy has completely changed the therapeutic armamentarium of patients with genitourinary tumors. In particular, the approval of anti-Programmed-death-1 (PD-1) agents in patients with renal cell carcinoma (RCC) and bladder cancer (BC) has increased their life expectancy, with a generally tolerated toxicity profile. On the contrary, patients with prostate cancer (PCa) seem to scarcely benefit from this strategy.

Due to the enthusiastic results obtained by immunocheckpoint inhibitors in cancer patients, researchers have increased their efforts to identify novel and more effective targets for immunotherapeutic approaches. Based on its pivotal activity, telomerase is emerging as a potential candidate for future therapeutic strategies in genitourinary tumors. Telomerase is a

ribonucleoprotein complex that maintains the length and integrity of telomeres, the ends of chromosomes, by restoring their repetitive sequences (TTAGGG) that would be lost during cell divisions [1]. Telomerase is overexpressed in about 80–95% of cancers and is present at very low levels or almost undetectable in normal cells [2]. Telomerase constitutes a complex system of large molecules that cooperate to maintain the chromosomal stability. They include three main components, called respectively human telomerase reverse transcriptase (hTERT), human telomerase RNA component (hTR) and telomerase associated protein 1 (TEP1) [3]. In addition, the two human telomeric repeat binding factors 1 and 2 (TRF1, TRF2) also contribute to telomerase activity [4]. Basically, hTR binds to the last few bases to 5'-3' strand and, successively, hTERT starts synthesizing new bases, leading to telomere lengthening and cell immortalization [5].

The expression of the telomerase degraded peptide fragments on tumor cell surface constitutes one of the means by which cancer cells are recognized and attacked by immune cells [6]. This is a standard event in cancer tissues and represents one of the key events that lead to the activation of cytotoxic T lymphocytes (CTL), thus promoting immune anti-tumor response.

This review emphasizes the role of telomerase in the carcinogenesis and progression of genitourinary tumors, thus underlying the potential of emerging telomerase-tailored immunotherapies in these patients.

## **2. Telomerase as a target for cancer immunotherapy**

Due to its key functions and its role as a hallmark of cancer cells, telomerase acts as an ideal target for cancer immunotherapy. These approaches include anti-telomerase vaccines and the transfer of hTERT-specific cytotoxic T lymphocytes.

### *2.1 Telomerase vaccination*

Telomerase-based vaccines are in course of evaluation in cancer patients. The majority of these vaccines are directed against hTERT, mostly due to its almost complete tumor specificity [7] and to

its ability to produce epitopes for both MHC class I and class II pathways [8]. At present, more than 25 different hTERT peptides have been exploited as epitope-mimicking structures or mimotopes. The majority of them are associated with MHC class I-mediated enhancement of CTL response, whilst a few of them are implicated in MHC class II-mediated induction of CD4<sup>+</sup> T cell response [9–12].

The list of vaccines includes several agents, such as GV1001 (in combination with Granulocyte Macrophage Colony-Stimulating-Factor, GM-CSF), GX301, GRNVAC1 and VX-001 [13] (Table 1). GV1001 consists of 16 amino acids and offers the advantage of eliciting both CD8<sup>+</sup> and CD4<sup>+</sup> responses [14,15]. It has been recently reported that GV1001 acts as a cell-penetrating peptide (CPP) allows the cytosolic delivery of macromolecules including proteins, DNA and siRNA through extracellular heat shock protein 90 (eHSP90) and 70 (eHSP70) complexes [16]. The eHSP-GV1001 complex may be taken up by antigen-presenting cells (APCs) and transferred to MHC class I molecules (cross-presentation), inducing a strong CTL response after recognition by CD8<sup>+</sup> T cells [17].

As for GX-301, it is composed by four peptides (peptide540-548, 611-626, 672-686 and 766-780), each one with the possibility to induce specific T cell responses [18]. It has been shown that GX301 to have anti-tumor activity and be well tolerated in patients with stage IV RCC or PCa resistant to conventional treatments. Indeed, prolonged Progression-Free Survival (PFS) and Overall Survival (OS) were observed, without serious adverse events [19].

On the other hand, GRNVAC1 (Table 1) is based on patient-derived dendritic cells pulsed with RNA encoding for a chimeric protein (lysosomal targeting signal LAMP fused to TERT) to enhance TERT peptide digestion and display [20]. Otherwise, VX-001 (Table 1) is composed by two peptides, ARG-Vx001 (TERT572) and TYR-Vx001 (TERT572Y). Vx-001 completed a randomized, placebo controlled, double blind phase IIb clinical trial in advanced NSCLC patients who did not progress after first line platinum based chemotherapy (NCT01935154). OS was longer

in the 29% of patients with a Vx-001 specific immune response (21.3 vs 13.4 months,  $p = 0.004$ ), and this advantage was stronger in never smokers [21].

## 2.2 Transferring *hTERT*-specific cytotoxic T lymphocytes

Another interesting immunotherapeutic approach targeting telomerase is based on engineering tumor-specific infiltrating lymphocytes (TILs). As for the other cells, extensive proliferation ultimately leads to a reduction of telomerase activity and to the shortening of telomeres in normal human T lymphocytes, eventually reaching a critically short length at which telomere function becomes compromised [2]. These events trigger cells to enter into a state of senescence that is characterized by altered functions and, notably, by the inability to further proliferate. On this scenario, it is possible to speculate that TILs with sufficient telomere length, due to a modified activity of telomerase itself, may avoid senescence and retain their antitumor activity *in vivo*. At this regard, several studies showed that the persistence of transferred TILs in patients' peripheral blood correlates with clinical response [21]. Interestingly, *hTERT*-transduced T cells have been shown to improve their proliferative potential, paving the way for an entirely new strategy in cancer immunotherapy, since these not-aging cells may be delivered to the patients to markedly prolong T-cell mediated immune response against tumor.

## 3. Renal Cell Carcinoma

### 3.1 Role of telomerase

It is well known that RCC is one of the most immune-responsive tumors and different immunotherapeutic approaches have been proposed in the management of this disease with variable degrees of success [22–25]. Among emerging immunotargets, telomerase represents one of the most promising due to several features including its high tumor-specificity. Indeed, *hTERT* mRNA levels resulted significantly higher in RCC compared to normal renal tissues and were significantly associated with higher tumor grade in clear cell RCC [26]. Telomerase complex has been associated

with the risk and progression of RCC. In particular, hTERT serum levels have correlated with tumor stage and distant metastases, while hTERT polymorphisms were linked to the risk of RCC [27]. Furthermore, incremented telomerase activity and reduced telomere length were proposed as predictive markers of RCC, while no correlation was reported with different histologic subtypes [28].

Interestingly, Hosen *et al.* investigated the presence of somatic activating mutations in the promoter region of hTERT in a series of 188 patients with clear cell RCC [29]. They showed that mutations in this region were not frequent and were associated with worst prognosis, particularly in the absence of the rs2853669 variant [29]. Similarly, Davis *et al.* revealed that genomic rearrangements leading to recurrent structural breakpoints in TERT promoter region were correlated with high TERT expression in a series of 66 patients with chromophobe RCC [30].

### 3.2 Targeting telomerase in RCC

In the last years, different approaches targeting telomerase complex have been proposed in RCC. These strategies include vaccine therapy, RCC-specific oncolytic immunovirotherapy and the use of chemotherapy or targeted agents. Although the lack of specific antigens has somehow limited the development of vaccine therapy in RCC, this approach is still of special interest in this disease. Currently, cell-based vaccines in RCC include autologous tumor-cell vaccines, genetically modified tumor vaccines and dendritic cell-based vaccines [31].

Several studies have been focused on testing the efficacy of RCC-targeted gene therapies. At this regard, Fang *et al.* assessed the antitumor activity of oncolytic adenoviruses equipped with a double siRNA targeting Ki67 and hTERT in 3 RCC cell lines, observing significant apoptosis of RCC cells *in vitro* and *in vivo* [32]. In the same view, the use of (hTERT)-controlled herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) resulted effective in inhibiting RCC cells in *in vitro* and *in vivo* models [33]. Furthermore, Huang *et al.* reported that the combination of Interleukine-2 (IL-2) and a telomerase-specific, replication-competent adenovirus (OBP-301) significantly

suppressed tumor growth and regulatory T-cells (Tregs) in a RCC mouse model [34]. Of note, the combination of shRNA gene therapy and oncolytic virotherapy can also synergistically enhance their antitumor efficacy in RCC cell lines [35].

Telomerase activity can be inhibited also by anti-Fas monoclonal antibody (mAb) in combination with doxorubicin, leading to synergistic cytotoxicity [36] or by MAP kinase inhibitors, which have been shown to induce cell death and reduce migration in RCC cell lines [26].

#### **4. Bladder cancer and upper tract urothelial carcinoma**

##### *4.1 Role of telomerase in the diagnosis and tumor recurrence*

Several evidences suggest that telomerase activity (in particular hTERT) is involved in the carcinogenesis of urothelial cancer [37]. BC cell lines have been shown to express each of the different telomerase subunits, whereas normal primary fibroblast cell lines express only hTERT and TEP1 mRNA but not hTERT mRNA [38]. hTERT promoter mutations have been identified in BC and upper tract urothelial carcinoma (UTUC), where they result more frequent than in RCC [39].

The specificity of hTERT expression on UC cells has supported its use as a biomarker for tumor diagnosis and recurrence. In 2003, Melissourgos *et al.* detected hTERT mRNA urinary expression in patients with malignant or benign urinary lesions as well in healthy subjects. They revealed that hTERT expression was found in over 90% of BC patients, with a sensitivity advantage against cytology in the tumor detection, particularly in low-grade lesions (93% vs 28%) [40]. Successively, Vinci *et al.* presented the results of a prospective study based on the quantitative methylation analysis of hTERT in urine sediment, supporting its potential use in the diagnosis of non-muscle invasive BC [41]. Of note, urinary content of hTERT was significantly correlated with subsequent tumor recurrence in patients with BC, with a higher sensitivity than urinary cytology [42]. In addition, hTERT may be useful to identify a subgroup of patients with superficial BC who will not benefit from Bacillus Calmette-Guérin (BCG) therapy and should be candidate for early salvage cystectomy [43].

#### 4.2 Targeting telomerase in urothelial cancer

In 2003, Kraemer *et al.* showed that antisense (AS)-oligonucleotides (ODNs) were able to specifically inhibit tumor proliferation in BC cell lines, as confirmed by reduced hTERT transcript amount protein content [44]. Successively, this group assessed whether the combination of hTERT AS-ODNs with common chemotherapy may increase drug-mediated antitumor activity in BC cell lines [45]. In this study, this combination increased the rate of apoptosis of BC cells and this event was associated with the activation of caspase 3 [45].

Interestingly, Zhou *et al.* observed that miR-1182 is deregulated in BC and its overexpression leads to the inhibition of tumor cell proliferation, colony formation and invasion [46]. They showed that miR-1182 directly targets hTERT by binding its 3'UTR and is highly implicated in modulating the chemosensitivity of BC cells [46].

Another promising strategies is based on small hairpin RNA (shRNA), which can inhibit the malignant phenotypes of tumor cell via ribonucleic acid interference (RNAi). At this regard, Lin *et al.* developed a tetracycline (Tet)- inducible small hairpin RNA that effectively suppressed hTERT and Bcl-2, thus inhibiting cell proliferation and migration and inducing apoptosis in BC cell lines 5637 and T24 [47].

### 5. Prostate cancer

#### 5.1 Role of telomerase in PCa

Despite its high antigen burden, PCa has not yet become an ideal “immunotherapy targeted” tumor. PROSTVAC is the only immunotherapeutic approach validated so far for this disease [48]. At the ASCO Annual Meeting 2018, Gulley *et al.* [49] have presented the results of the PROSPECT study, a phase III trial enrolling 1297 patients with asymptomatic or minimally symptomatic metastatic PCa randomized to receive PROSTVAC or PROSTVAC plus Granulocyte-macrophage Colony-

Stimulating Factor (GM-CSF) or placebo. The authors have reported no significant differences in terms of OS among the three treatment arms, not confirming the results previously observed [48]. However, several evidences suggest that PCa will have a much more rich future in the upcoming immunotherapy era. Indeed, the recent results presented at the ASCO Annual Meeting 2018 have suggested that anti-PD-1 agent Pembrolizumab has antitumor activity with a safe toxicity profile in patients with docetaxel-refractory metastatic castration-resistant PCa, regardless of PD-L1 status [50]. In this sense, targeting telomerase complex via immune-mediated strategies could represent one of the most interesting strategies. This consideration moves from the fact that, as for the majority of tumors, hTERT appears to be upregulated in PCa than in normal prostate cells [49] (Figure 2). Moreover, there are several other molecules involved in the deregulated “telomerase mechanism” in PCa, such as TRF1 and TRF2 [52], suggesting their potential role in future therapeutic strategies aimed to disrupt the telomerase oncogenic activity.

### 5.2 Targeting telomerase in PCa

As mentioned above, one of the reasons why immunotherapy could target hTERT is certainly linked to the high reactivation rate of this molecule observed in PCa tissues. Following this suggestion, Lilleby *et al.* led a phase I/IIa study to assess the efficacy and safety of a novel hTERT peptide vaccine (UV1) in patients with hormone-naïve PCa [53] (Table 1). In this trial, immune responses against UV1 peptides were reported in 85% of patients, with 64% showing PSA declined to <0.5 ng/mL and 45% with no radiologic evidence of persisting disease in the prostatic gland [53]. In 2005 Su and his group investigated a completely new approach to treat PCa, using Telomerase mRNA-Transfected Dendritic Cells (DC) [54]. In a previous study, DC transfected with mRNA encoding Lysosome-associated membrane protein-1 (LAMP) hTERT protein showed the ability to stimulate CD4<sup>+</sup> T cell and hTERT-specific CD8<sup>+</sup> T cell responses *in vitro* [54]. On this basis, mRNA-transfected dendritic cells (DC) were administered to 20 patients with metastatic PCa. The subjects were randomized to receive hTERT mRNA-transfected DC or LAMP hTERT mRNA-

transfected DC; then they were also randomized to receive three or six intradermal injections of these two preparations. The vaccines were given intradermally and patients underwent a leukapheresis to obtain useful DC. Then, DCs were generated starting from monocytic precursors stimulated through IL-4 and GM-CSF. Once obtained a sufficient amount of DC, these were transfected with hTERT or LAMP hTERT mRNA. A common reaction observed among patients treated with DC vaccines (all but two patients) was grade I inflammatory response at the site of injection, with a deep infiltration of CD4<sup>+</sup> T cells. A subsequent analysis of peripheral blood of study subjects showed a consistent number of hTERT-specific CD4<sup>+</sup> T cell. Interestingly, vaccine-induced CD4<sup>+</sup> T cell exhibited a consistent lytic activity against hTERT-expressing targets. On the clinical side, this study suggests that vaccination with hTERT mRNA-transfected DC is associated with a certain, even if short-lasting, impact on PSA doubling time and with a transient reduction of circulating tumor cells. Both these effects can be considered as surrogate markers of clinical response and were closely associated with hTERT-specific T cell activity.

A novel method for the production of therapeutic dendritic-like cells called Tumor Antigen Presenting Cells (TAPCells) has been also investigated in PCa patients (Table 1). In a phase I trial led by Reyes *et al.* [55], a significant decrease in PSA serum levels was reported in 6 of the 14 patients enrolled, suggesting that this approach should be further investigated in larger clinical trials.

A totally different way to interact with telomerase activity in PCa is targeting this molecule in the tumor-initiating cells (TICs) [56]. TICs are considered to be a pool of cells responsible developing relapses and distant metastasis. TICs have specific surface markers, such as CD44 and CD133 [57], which allow identifying and isolating them from a panel of PCa cell lines. Very notably, most of PCa tissues have a consistent amount of TICs with a strong telomerase activity, supporting the investigation of telomerase inhibitor drugs in this setting.

Several studies used a novel compound named Imetelstat sodium (GRN163L) in order to block telomerase activity in TICs. GRN163L is a N3'-P5 thio-phosphoamidate oligonucleotide

antagonist, known as one of the most efficient telomerase inhibitor so far used both in preclinical and in preliminary clinical trials [58]. GNR163L has an important telomere shortening activity in several cancer cell lines [59,60]. This oligonucleotide works by interacting with hTR template region of telomerase. In the Marian's study GRN163L was given at TCIs isolated from PCa samples. Then, the telomerase activity in TCIs was measured using specific PCR reactions; in particular was used a protocol called TRAP (Telomeric Repeat Amplification Protocol). The results of the study showed an important telomere shortening in TICs treated with GRN163L. Furthermore, the long-term treatment with GRN163L may significantly reduce the count of TICs and lead to a decrement in terms of self-renewal attitude in PCa cell lines. These preliminary findings suggest that telomerase inhibition by this brand new class of small molecules, along with standard treatments already known, may determine a stronger and durable response in patients with PCa, opening an entirely new perspective in the field of cancer therapy research.

## 6. Discussion

The research for novel effective immunotargets has identified telomerase complex as an ideal candidate for the treatment of genitourinary tumors. This is the result of a long process that has seen the progressive introduction of novel approaches, such as adoptive cell transfer (ACT) and new techniques for vaccine development, into the armamentarium of cancer researchers.

The presence of hTERT peptide epitopes on tumor cells [61] support the notion that hTERT represents an attractive target for cancer immunotherapy. Consistently, *in vitro* and *in vivo* studies have shown effective anti-tumor activity of hTERT-tailored strategies in genitourinary tumors, including active immunotherapy with hTERT-peptide vaccines and passive immunotherapy with hTERT-transduced T cell infusion. Increasing the specificity and efficacy of hTERT-targeted immunotherapy still represents a major focus in cancer research. A new generation of cancer vaccine for hTERT-targeted immunotherapy needs the identification of novel highly hTERT-

specific tumor-associated antigens in order to increase the vaccine specificity and ability to kill hTERT<sup>+</sup> tumor cells.

On the scenario of a day-by-day increasing use of immuncheckpoint inhibitors and other agents aimed at enhancing T cell anti-tumor response in genitourinary tumors, identifying the most effective sequential or combined approaches represents the next goal for cancer researchers. In order to increase the drug-induced anti-tumor activity of immune cells, prolonging T cell survival is rapidly becoming one of the most interesting approaches and has lead to the definition of “living drugs” in cancer therapy. Among emerging techniques to enhance T cell survival, hTERT-transduced T cells and T-CAR seem to represent, at present, the most promising strategies. As mentioned above, hTERT-transduced T cells are not-aging cells characterized by improved proliferative potential, supporting the hypothesis that combining this approach with immuncheckpoint inhibitors may lead to a synergic immune mediated response against tumor. In the same view, CAR-T technique, based on engineering T cells to express chimeric antigen receptors (CARs) that recognize cancer-specific antigens, has evolved from its first to third generation by the introduction of a series of multiple signaling components, such as CD3z-CD28-CD137 (4-1BB) or CD3z-CD28-CD134 (OX40), to increase T cell survival and prolong their anti-tumor effects. In this context, CAR-T cells after the transient delivery of modified TERT mRNA increased proliferation and delayed senescence [62] and, as a consequence, their long-term antitumor activity in mouse xenograft tumor models.

## 7. Conclusions

Immunotherapy targeting telomerase can be expected to continuously expand fast in the next years. However, further clinical trials are needed to build a broader base for anti-telomerase cancer therapy and to integrate it within the immunological treatments of cancer. A better understanding of tumor-host interactions and of intracellular trafficking, processing and presentation of hTERT antigen will be crucial for the development of a new era of immunotherapeutic agents.

**Conflict of Interest Statement**

We declare to have no conflict of interest.

ACCEPTED MANUSCRIPT

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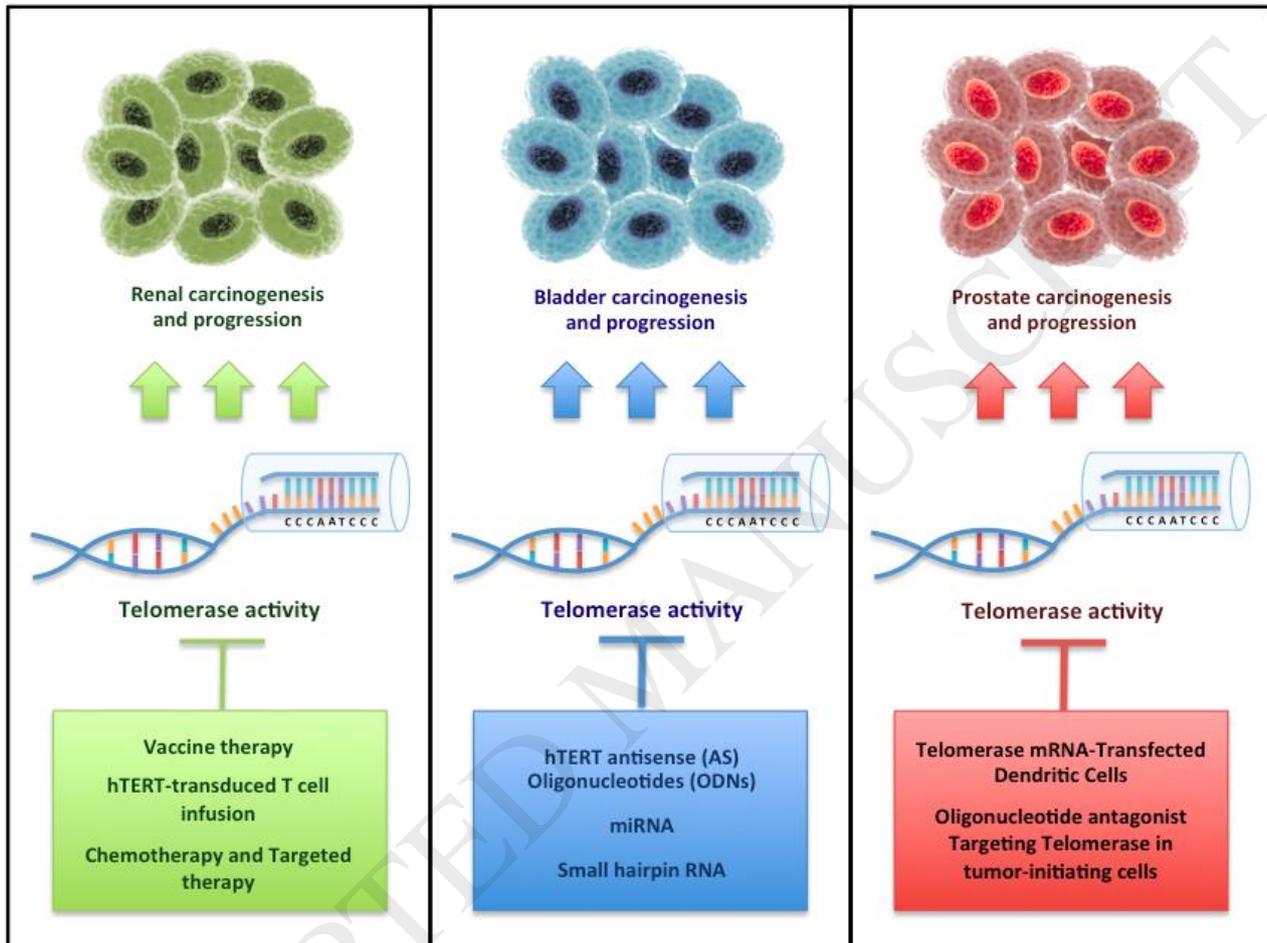
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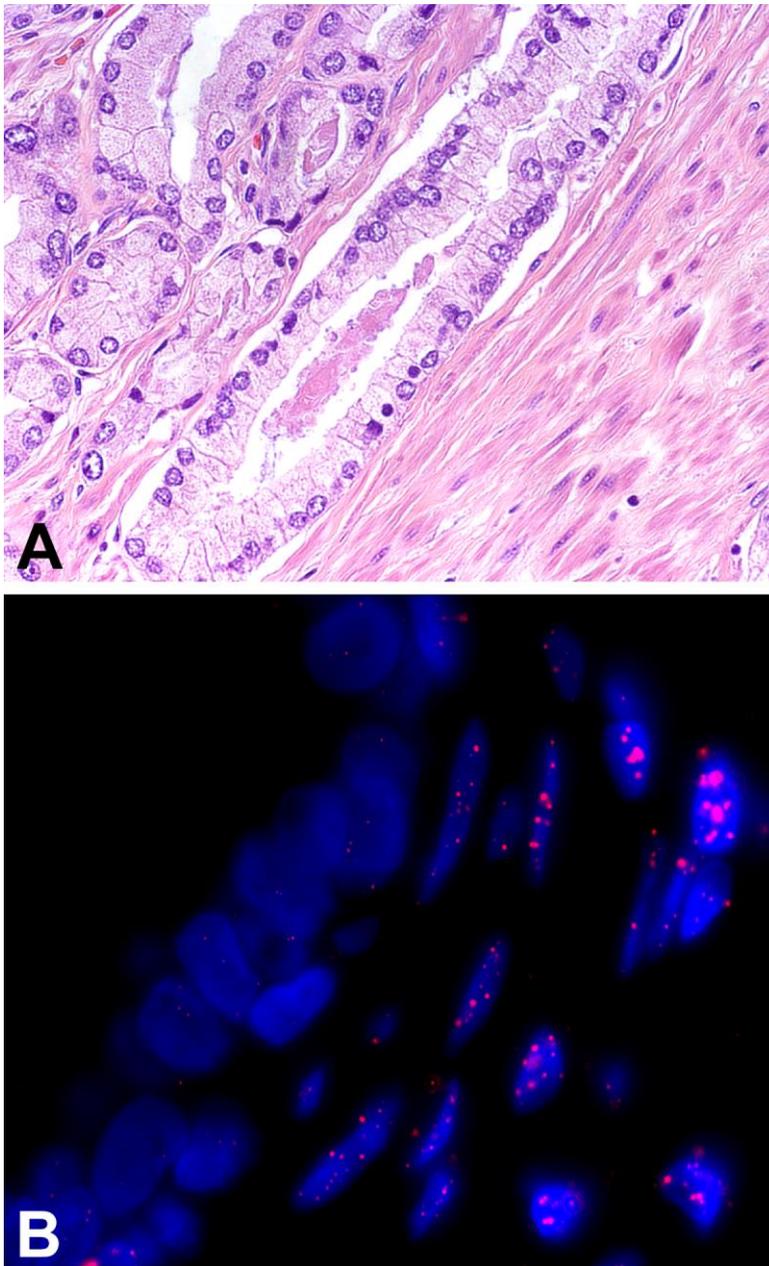
## Table Legends

## Figure Legends

**Figure 1.** Telomerase-tailored strategies in genitourinary tumors. hTERT = human telomerase reverse transcriptase; miRNA = microRNA.



**Figure 2.** FISH with telomere-specific probe of showing reduced telomere signal intensity in PCa cells [B] compared with the adjacent stromal cells [A].



**Table 1.** Agents targeting telomerase and mechanisms of action.

<b>Agent</b>	<b>Target</b>	<b>Therapeutic approach</b>	<b>Mechanism</b>
GV1001	hTERT	Vaccine and Cell-Penetrating Peptide (CPP)	Induction of a strong CTL response after recognition of the eHSP-GV1001 complex transferred to MHC class I by CD8+ T cells
GX301	hTERT	Vaccine	Composed by four peptides, each one with the possibility to induce specific T cell responses
GRNVAC1	hTERT	Vaccine	Patient-derived dendritic cells pulsed with RNA encoding for a chimeric protein to enhance TERT peptide digestion and display
VX-001	hTERT	Vaccine	Induction of CD4 and CD8 immune response
UV1	hTERT	Vaccine	Induction of CD4 and CD8 immune response
TAPCells	hTERT	Vaccine	Induction of CD4 and CD8 immune response
GRN163L	hTR	N3'-P5 thio-phosphoamidate oligonucleotide antagonist	Reduction of tumor-initiating cells