Telomerase as a new target for the treatment of hormone-refractory prostate cancer

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Abstract

Prostate cancer is the leading cause of cancer-related deaths in men. Androgen ablation is the mainstay of treatment for advanced prostate cancer. This therapy is very effective in androgen-dependent cancer; however, these cancers eventually become androgen independent, rendering anti-androgen therapy ineffective. The exploration of novel modalities of treatment is therefore essential to improve the prognosis of this neoplasm.

Telomeres are specialized heterochromatin structures that act as protective caps at the ends of chromosomes. Telomere maintenance in the majority of tumor cells is achieved by telomerase, a reverse transcriptase enzyme that catalyzes the synthesis of further telomeric DNA. Telomerase is detected in the majority of prostate cancers, but not in normal or benign prostatic hyperplasia tissue. Moreover, the human telomerase reverse transcriptase (hTERT) gene, the catalytic subunit of telomerase, is regulated by androgens as well as by different oncogenes including Her-2, Ras, c-Myc and Bcl-2, which seem to play an important role in prostate cancer progression. Thus, telomerase may represent a very good candidate for targeted therapy in prostate tumors. To inhibit telomere maintenance by telomerase, approaches that directly target either telomerase and telomeres or the telomerase regulatory mechanisms have been used. Moreover, strategies targeting telomerase-positive cells as a means to directly kill the tumor cells have been tested. This review summarizes the most promising results achieved by anti-telomerase strategy in different solid tumors. Most of the telomerase-associated therapies described here have proved very promising for the treatment of prostate cancer. On the basis of the good results obtained and considering the multigenic defects of human tumors, including prostate cancer, the combination of anti-telomerase strategies with conventional drugs and/or molecules capable of interfering with oncogenic pathways could efficiently improve the response of this neoplasm.

Introduction

Prostate cancer is the most frequently diagnosed malignancy, apart from skin cancers, in developed countries, and is a leading cause of cancer-related deaths in men (Feldman & Feldman 2001). The risk of prostate cancer rises with age and continues to increase by 3–4% each year as fewer men die from cardiovascular disease. Androgen ablation is the leading treatment and currently the most successful for progressive prostate cancer. However, due to the emergence of androgen-independent cells, tumors have become insensitive to this kind of treatment (Denmeade & Isaacs 2002). Continuous effort is therefore required to find new targets for therapeutic intervention of androgen-independent prostate cancer.

One of the most common causes by which prostate tumors have become androgen independent is the amplification of the androgen receptor gene. It has been reported that about 30% of tumors exhibiting androgen independence after ablation therapy shows overexpression of the androgen receptor (Koivisto et al. 1997, Linja et al. 2001). Amplified levels of the androgen receptor are...
accompanied by an increased sensitivity to the androgens. The dose of androgen necessary to stimulate cell proliferation in androgen-independent cancer cells is therefore significantly lower than that observed in androgen-dependent cancer cells (Culig et al. 1999, Gregory et al. 2001a).

The switch from androgen-dependent to -independent tumors has also been associated with an increase in the mutation frequency of the androgen receptor. When the androgen receptor is wild type it is specifically activated by testosterone and dihydrotestosterone, while androgen receptor mutations give broadened ligand specificity and the mutated receptor can also be stimulated by glucocorticoids and flutamide (Taplin et al. 1999, Zhao et al. 2000, Hara et al. 2003).

The transcriptional activity of the androgen receptor is normally modulated by co-regulatory proteins. It has been reported that the onset of recurrent prostate cancer growth after androgen ablation therapy might result from the overexpression of coactivators, such as the two nuclear receptor coactivators, transcriptional intermediary factor 2 and steroid receptor coactivator 1 (Gregory et al. 2001b). Overexpression of these coactivators increases androgen receptor transactivation and enhances responses to low levels of androgen.

A number of molecular genetic changes have also been associated with the increased proliferation and/or apoptosis inhibition in prostate tumors in the absence of androgens or the androgen receptor. In particular, it has been demonstrated that Her-2 overexpression enhances the interaction between the androgen receptor and coactivators, thus indicating a role for aberrant expression of Her-2 in prostate carcinogenesis as well as progression to androgen-independent growth (Yeh et al. 1999). Constitutive activation of the Ras/MAP kinase pathway correlates with hormone refractory tumor growth (Bakin et al. 2003) and it has been associated with advanced grade and stage of prostatic tumors (Gioeli et al. 1999). Inhibition of apoptosis by bcl-2 oncogene is suggested as another mechanism by which prostate tumors escape from androgen ablation therapy (Miyake et al. 1999, Bernard et al. 2003). Negative staining of Bcl-2 was found in benign prostatic hyperplasia, while Bcl-2 positivity was observed in premalignant prostatic intra-epithelial neoplasia, which is considered the primary precursor of human prostate cancer (McDonnell et al. 1992, Raffo et al. 1995). Finally, overexpression and/or amplification of the c-myc gene have been observed in about 70% of androgen-independent prostate cancers (Nupponen et al. 1998, Nupponen & Visakorpi 2000). The c-Myc-dependent prostate cancer cell proliferation appears to be androgen independent, since the reduction of androgen receptor expression is ineffective in prostate tumors overexpressing c-Myc (Bernard et al. 2003). All these findings indicate that beyond the alterations in the androgen-dependent pathway, other mechanisms are involved in prostate cancer progression.

Telomerase in prostate cancer

Telomeres are specialized heterochromatin structures that act as protective caps at the ends of chromosomes. Human telomeres consist of tandem repeats of the hexanucleotide sequence TTAGGG in double strand, except for a terminal 3' G-rich overhang (McElligot & Wellinger 1997, Makarov et al. 1997, Wright et al. 1997). In normal somatic cells, telomeres shorten with each round of cell division and, when they reach a critically short length, cells exit from the cell cycle and undergo a replicative senescence. By contrast, immortal cells as well as germine cells adopt some mechanisms to bypass the senescence checkpoint. Telomere maintenance in 80–95% of tumor cells is achieved by telomerase, a reverse transcriptase enzyme that catalyzes the synthesis of further telomeric DNA repeats.

The telomerase holoenzyme consists of the catalytic subunit reverse transcriptase protein hTERT (Nakamura et al. 1997), the telomerase RNA template subunit, hTR (Feng et al. 1995) and other associated proteins (Harrington et al. 1997).

Telomerase activity is typically absent from most normal human cells, but is expressed in nearly all human cancer cells (Kim et al. 1994, Broccoli et al. 1995, Hiyama et al. 1995a). In prostate tissue, telomerase activity has not been found in normal samples or benign prostatic hyperplasia tissues, while primary prostate cancers exhibit high levels of telomerase activity (Sommerfeld et al. 1996, Lin et al. 1997). In agreement with these results, hTERT immunoreactivity was found in high-grade prostate tumors (Gleason score >4) (Iezkowski et al. 2002). Since it has been demonstrated that androgen depletion activated telomerase in the prostate of monkeys, the negative regulation of telomerase activity by androgen is probably lost during prostate tumorigenesis (Ravindranath et al. 2001).

Telomerase activity has also been correlated with prostate tumor aggressiveness. In particular, compared with low grade tumors, high grade tumors have maximally activated telomerase and a significant correlation between the telomerase activity and the Gleason score has been found (Kamradt et al. 2003). Interestingly, the hTERT gene seems to be regulated by androgens. Administration of androgens to androgen-sensitive LNCaP prostate cancer cells activated the hTERT promoter, while androgen ablation led to a decrease in hTERT expression with a concomitant reduction in telomerase activity which, in turn, was reversed by the
subsequent administration of androgens (Bosland 2003). Consistent with these results, a decline in hTERT immunostaining has been observed in human specimens of prostate cancer after complete ablation therapy and the steepest reduction in telomerase activity was noted in the highest grade tumors (Iczkowski et al. 2004). Moreover, the hTERT gene is regulated by different oncogenes including c-Myc (Wang et al. 1998), Bcl-2 (Mandal & Kumar 1997), Her-2 and Ras (Goueli & Janknecht 2004), which seem to play an important role in prostate cancer progression.

All these findings suggest that telomerase might represent a very good candidate for targeted therapy in prostate tumors. Finally, considering the multigenic defects of human tumors, including prostate cancer, the combination of anti-telomerase strategies with conventional drugs and/or molecules capable of interfering with oncogenic pathways might efficiently improve the response of this neoplasia.

This review summarizes the most promising results achieved by anti-telomerase strategies in different tumors, including prostate cancer.

**Inhibiting telomerase**

To inhibit telomere maintenance by telomerase, approaches that directly target either telomerase and telomeres or the telomerase-associated regulatory mechanisms have been used (Fig. 1). Moreover, strategies
targeting telomerase-positive cells as a means of directly killing the tumor cells were tested.

Approaches targeting telomerase and telomeres

Telomeric DNA and the core telomerase components of telomerase, hTR and hTERT, are definitely required for telomerase function and therefore they are good targets for anti-telomerase strategy. In Table 1 the most promising approaches used to directly target telomerase and telomeres are shown.

2'-5' oligoadenylate antisense

2'-5' oligoadenylate antisense (2–5A) strategy has been applied to specifically and efficiently degrade hTR (2–5A anti-hTR). The 2–5 anti-hTR is a chimeric antisense oligonucleotide obtained by linking a 19-mer antisense oligonucleotide targeting hTR to a 2–5A molecule able to recruit and activate the endoribonuclease RNAse L at the site of the target RNA sequence. The treatment of human prostate cancer cells with 2–5A anti-hTR efficiently reduced the cell viability in a very short time exposure (Kondo et al. 2000). Similar anti-tumoral effects following treatment with 2–5A anti-hTR were observed in other telomerase-positive tumors, such as malignant glioma, ovarian, bladder and cervical cancer cells (Komata et al. 2000, Kushner et al. 2000, Mukai et al. 2000, Koga et al. 2001a, Yatabe et al. 2002). Interestingly, telomerase-negative astrocytes, endothelial cells or fibroblasts are resistant to the 2–5A anti-hTR treatment. The efficacy of 2–5A anti-hTR has also been evaluated in vivo. The results demonstrated that intra-tumoral treatment with 2–5A anti-hTR of prostate, glioma and bladder xenografts produced a significant anti-tumor effect due to apoptosis induction (Kondo et al. 1998, Mukai et al. 2000, Koga et al. 2001). Moreover, a synergistic anti-tumoral effect was observed by combining the 2–5A anti-hTR with the anti-cancer drug cisplatin or the recombinant adenovirus carrying the p53 tumor suppressor (Komata et al. 2000, Kondo et al. 2001). Therefore, 2–5A anti-hTR represents an effective novel approach for the treatment of telomerase-positive tumors including prostate cancer as a single or combined treatment.

N3'–P5' phosphoramidate oligonucleotides

N3'–P5' phosphoramidate oligonucleotides (NP) have been designed, synthesized and evaluated as telomerase inhibitors. NP oligonucleotides were targeted at a segment of hTR downstream from the hTR template region, but

<table>
<thead>
<tr>
<th>Target</th>
<th>Therapeutic approach</th>
<th>Tumor type</th>
<th>Molecular effects</th>
<th>Biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomerase hTR</td>
<td>2'-5' oligoadenylate antisense</td>
<td>Glioma, prostate, ovarian, bladder and cervical cancers</td>
<td>Telomerase inhibition</td>
<td>Apoptosis, increased sensitivity to cisplatin</td>
</tr>
<tr>
<td></td>
<td>N3'–P5' thio-phosphoramidate oligonucleotides (GRN163)</td>
<td>Prostate cancer</td>
<td>Telomerase inhibition, telomere shortening</td>
<td>Apoptosis, senescence</td>
</tr>
<tr>
<td></td>
<td>Ribozymes</td>
<td>Melanoma</td>
<td>Telomerase inhibition</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Peptide nucleic acids</td>
<td>Melanoma and prostate cancer</td>
<td>Telomerase inhibition, telomere shortening</td>
<td>Growth arrest, apoptosis, apoptosis, inhibition of proliferation, apoptosis, increased sensitivity to topoisomerase inhibitors</td>
</tr>
<tr>
<td>hTERT</td>
<td>Ribozyme</td>
<td>Breast and ovarian cancers</td>
<td>Telomerase inhibition, telomere shortening</td>
<td>Reduced cell survival, Growth arrest, apoptosis, increased sensitivity to cisplatin</td>
</tr>
<tr>
<td></td>
<td>Peptide nucleic acids</td>
<td>Prostate cancer</td>
<td>Telomerase inhibition, telomere shortening</td>
<td>Senescence-like growth arrest, Growth arrest, increased sensitivity to paclitaxel</td>
</tr>
<tr>
<td></td>
<td>Dominant-negative</td>
<td>Melanoma, breast, ovarian and colon cancers</td>
<td>Telomerase inhibition</td>
<td>Senescence-like growth arrest, Growth arrest, increased sensitivity to paclitaxel</td>
</tr>
<tr>
<td></td>
<td>Small molecule (BIBR19532)</td>
<td>Prostate, lung and breast cancers</td>
<td>Telomerase inhibition, telomere shortening</td>
<td>Senescence-like growth arrest, Growth arrest, decreased sensitivity to paclitaxel</td>
</tr>
<tr>
<td>Telomeric G-quadruplex</td>
<td>G-quadruplex ligand (Acridine compounds)</td>
<td>Breast and vulval cancers</td>
<td>Telomerase inhibition</td>
<td>Senescence-like growth arrest, Growth arrest, increased sensitivity to paclitaxel</td>
</tr>
</tbody>
</table>
they did not activate RNase-H. Thus, these compounds are not true antisense oligonucleotides, but rather behave like classical active-site enzyme inhibitors, i.e. 'template antagonists'. NP oligonucleotides were employed because they form very stable duplexes with single-stranded RNA, are resistant to nuclease and display a high affinity for nucleic acids, but not proteins. Preliminary results indicated that these oligos do not inhibit telomerase activity in vivo (Pruzan et al. 2002). The same group then tested the anti-telomerase effect of N3'→PS' thio-phosphoramidate oligonucleotides (NPS). Telomerase inhibition occurred 24 h after transfection of an immortalized human breast epithelial cell line at submolar concentrations (Herbert et al. 2002). Long-term treatment with NPS oligonucleotides resulted in gradual telomere shortening followed by cellular senescence and apoptosis, while the mismatched control compound had no effect on cell proliferation (Herbert et al. 2002). Optimization of the sequence, length and bioavailability resulted in the selection of the novel telomerase template antagonist, GRN163 (Asai et al. 2003). GRN163 inhibited telomerase in various tumor cell lines in the presence and absence of carriers. Tumor cells treated with GRN163 in culture underwent telomere shortening and senescence or apoptosis after a period of time and this generally correlated with initial telomere length. In a prostate cancer xenograft model, parentally administered GRN163 caused suppression of tumor growth. These experiments provided evidence that modified antisense oligonucleotides complementary to hTERT could be effective anti-tumoral agents for prostate cancer.

Hammerhead ribozymes

Hammerhead ribozymes are catalytic antisense RNAs that cleave RNA substrates in a sequence-specific manner. A number of studies on experimental human tumor models have demonstrated the possibility of efficiently inhibiting telomerase activity through the use of ribozyme targeting hTERT (Yokoyama et al. 1998, Folini et al. 2002). However, changes in cell proliferation or induction of apoptosis were not always observed.

Hammerhead ribozymes targeting the mRNA of the catalytic telomerase component hTERT have also been generated (Yokoyama et al. 2000, Ludwig et al. 2001, Saretzki et al. 2001). The results demonstrated the ability of these molecules to cleave hTERT mRNA and inhibit telomerase activity in endometrial, breast and ovarian carcinoma cells. Ribozyme-mediated reduction of telomerase activity resulted in inhibition of cell proliferation and induction of apoptosis with or without telomere erosion. Moreover, an increased sensitivity to topoisomerase inhibitors was also observed, suggesting the possibility of combining the ribozyme approach for antitelomerase cancer strategy with chemotherapy.

Peptide nucleic acids

Peptide nucleic acids (PNAs) are modified oligonucleotides that contain a non-ionic backbone in which the deoxyribose linkages have been replaced by N-(2-aminoethyl) glycin units, making them resistant to degradation. PNAs complementary to both core telomerase components were generated. Norton and coworkers (1996) demonstrated the possibility of inhibiting telomerase activity in cell extract by PNAs complementary to the hTR. However, because the membrane permeability of PNAs is not high, strategies to improve the in vivo uptake have been used. In particular, PNAs have been delivered into intact cells by electroporation, generation of PNA-DNA complexes or conjugation with transport peptides (Herbert et al. 1999, Shammas et al. 1999, Villa et al. 2000). The treatment of immortal human cells with hTR PNAs was able to reduced telomerase activity, shortened telomeres, reduced colony size and arrested cell proliferation (Shammas et al. 1999). PNA-mediated inhibition of telomerase activity, followed by growth arrest or apoptosis, was also demonstrated in human prostate and melanoma cells (Herbert et al. 1999, Villa et al. 2000).

Recently, PNAs targeting the catalytic subunit hTERT were generated (Huard & Autexier 2002). hTERT-PNAs were delivered into the cytoplasm of human prostate cancer through the photochemical internalization and HIV-Tat protein-based approaches. After light exposure, cells treated with hTERT-PNA showed a marked inhibition of telomerase activity and a reduced cell survival, which was not observed after treatment with hTERT-PNA alone. Moreover, in a direct comparison, photochemical internalization technology proved to be more efficient at internalizing the hTERT-PNA than an HIV-Tat protein-based approach.

Dominant-negative hTERT

Dominant-negative hTERT (DN-hTERT) constructs are mutated at critical amino acid residues within the catalytic domain and virtually abolished telomerase activity by competing for binding to other components of the telomerase holoenzyme complex that are essential for catalysis. Two independent groups (Hahn et al. 1999, Zhang et al. 1999) have demonstrated the ability of DN-hTERT constructs to inhibit telomerase activity in immortalized and tumor cells. Loss of telomerase activity in various human cancer cell lines of different histotypes led to a gradual reduction in telomere length followed by growth arrest or apoptosis, whereas wild-type hTERT transfected cells did not show any changes in telomere length and cell proliferation. Moreover, telomerase-inhibited human cell lines were unable to form tumors when injected into immunodeficient nude mice, validating telomerase as an important therapeutic target.
Recently, it has been demonstrated that inhibition of telomerase using the DN-hTERT construct not only limits the growth of tumor cells, but also sensitizes them to anti-neoplastic drugs (Misawa et al. 2002, Tauchi et al. 2002, Tentori et al. 2003). In this context, we recently used the DN-hTERT construct in order to demonstrate the involvement of telomerase in the chemosensitivity of melanoma cells. First, we demonstrated that inactivation of telomerase in melanoma cells, by expressing the DN-hTERT construct, induced telomere shortening followed by cellular crisis, demonstrating the role of telomerase on this tumor histotype (Biroccio et al. 2003a). Moreover, inhibition of telomerase increased resistance to temozolomide and increased sensitivity to cisplatin, while there was no change in the response to tamoxifen, indicating that a careful selection of anti-neoplastic agent has to be made when anti-telomerase therapy is combined with chemotherapy (Tentori et al. 2003).

**Small molecules**

Nucleoside analogs bind the nucleotide-binding site of reverse transcriptase enzyme and are incorporated into the DNA during the elongation step, thereby interfering with the incorporation of dNTPs. Nucleoside analog inhibitors such as 3-azido-2’,3’-dideoxynucleoside (AZT) were among the first synthetic compounds tested against telomerase. However, they only exhibit weak inhibitory activity for human telomerase and mild anti-proliferative capacity (Strahl & Blackburn 1996, Melana et al. 1998). Chronic treatment of breast cancer cells with AZT induced a senescent phenotype and reduced tumorigenicity only at very high concentrations (Tejera et al. 2001). Novel derivatives of 7-deaza-2’-deoxypurine nucleoside triphosphate are much more potent inhibitors of telomerase in vitro (Fletcher et al. 2001).

Non-nucleoside inhibitors bind the hydrophobic pocket near the enzyme’s catalytic center, resulting in a conformational change in the active site. BIBR1532 is a novel non-nucleosidic telomerase inhibitor highly specific for telomerase, since it does not seem to affect other DNA and RNA polymerases (Damm et al. 2001). Treatment with BIBR1532 of different human tumor cells, including prostate cancer, led to a progressive telomere shortening followed by senescence-like growth arrest. Telomerase inhibition and telomere shortening also resulted in a marked reduction of the tumorigenic potential of treated cells in a mouse xenograft model.

**G-quadruplex DNA-interactive compounds**

DNA sequences that contain stretches of guanine can form four-stranded structures called G-quadruplexes. Potential G-quadruplex-forming sequences are found at telomeres and extend as a single-stranded G-rich 3’-overhang. Molecules capable of interacting with four-stranded G-quadruplex have been generated. These agents are capable of stabilizing the G-quadruplex structure, inhibiting access of telomerase to the telomeres and blocking the elongation step catalyzed by telomerase (Zahler et al. 1991, Mergny et al. 1999). Classes of G-quadruplex inhibitors described to date include antracyclines (Sun et al. 1997, Perry et al. 1998a,b), flourenones (Perry et al. 1999a), acridines (Harrison et al. 1999), cationic porphyrines (Izbicka et al. 1999, Shi et al. 2001), a perylenetetra-carboxylic di-imide derivative (Fedoroff et al. 1998), indolo-quinolines (Caprio et al. 2000) and a benzonaphthofurandione tetracyclic compound (Perry et al. 1999b). However, the development of these G-quadruplex-interacting molecules is severely limited by the relatively poor selectivity for binding to quadruplex versus duplex DNA. This is reflected in these compounds because they showed acute cytotoxicity at concentrations similar to those required for telomerase inhibition. Recently, some other series of potent G-quadruplex-interactive telomerase inhibitors based on ethidium (Koeppel et al. 2001), dibenzenophenanthrolines (Mergny et al. 2001), pentacyclic acridines (Gowan et al. 2001, 2002), quinoline (Riou et al. 2002) and the microbial product telomestatin (Shin-ya et al. 2001) have been described. The acridine compounds RHSP4 and BRACO19 are potent cell-free inhibitors of human telomerase and they do not cause non-specific acute cell cytotoxicity at similar concentrations to those required to completely inhibit telomerase activity (Gowan et al. 2001, 2002). The exposure of human cancer cells with short telomeres to non-acute cytotoxic concentrations of both compounds resulted in a marked reduction in cell growth. Moreover, significant anti-tumor activity in vivo was observed when BRACO19 was administered after paclitaxel to mice bearing an advanced stage of vulval carcinoma (Gowan et al. 2002).

**Approaches targeting the telomerase regulatory mechanisms**

Recent studies on telomerase regulation showed that telomerase activation is achieved at various steps, including transcriptional and post-transcriptional levels of the hTERT. A number of transcriptional factors, cell cycle inhibitors, cell fate determining molecules, hormone receptors and viral proteins have been implicated in the control of hTERT. Some of the hTERT expression regulators and the main approaches used to inhibit them are reported below and in Table 2.

**Transcriptional factor inhibitors**

A variety of transcriptional factors have been shown to participate in hTERT gene expression (Poole et al. 2001).
Neuroblastomas and cervical cancers (Hiyama et al. 2000) expression correlates with Myc overexpression in prostate, has also been observed in tumors. In particular, hTERT expression, telomerase activity and telomere shortening were demonstrated that the downregulation of c-Myc reduced the susceptibility to cisplatin and to the novel anti-cancer drug eteinascidin-743. Reconstitution of hTERT in the c-Myc low-expressing clones restored telomerase activity, reduced telomere dysfunction and decreased the sensitivity to both drugs, by enabling the cells to recover from drug-induced G2/M block and consequently protecting them from apoptosis. Together these findings clearly demonstrated that inhibition of c-Myc oncoprotein represents an effective strategy for the treatment of telomerase-positive tumors as a single or combined treatment.

Demethylating agents

5-Azacytidine is a demethylating agent able to induce gene activation by reverting hypermethylation of altered genes (Jones & Taylor 1980). The resulting DNA hypomethylation has been linked to the induction of cellular differentiation and altered expression of genes involved in tumor suppression (Herman et al. 1994, 1995, 1996). Demethylating agents have therefore been used as potential anti-neoplastic drugs for some types of tumors, including prostate cancers (Prasanna et al. 1995).

Table 2 Targeting of the telomerase regulatory mechanisms.

<table>
<thead>
<tr>
<th>Target</th>
<th>Therapeutic approach</th>
<th>Tumor type</th>
<th>Molecular effects</th>
<th>Biological effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcriptional factors</td>
<td>Gene transfer</td>
<td>Melanoma</td>
<td>hTERT and telomerase inhibition, telomere shortening</td>
<td>Apoptosis, crisis, increased sensitivity to cisplatin and eteinascidin-743</td>
</tr>
<tr>
<td>c-Myc</td>
<td>Chromatin</td>
<td>Prostate cancer</td>
<td>hTERT and telomerase inhibition</td>
<td>Inhibition of proliferation</td>
</tr>
<tr>
<td></td>
<td>Demethylating agents</td>
<td>Liver and prostate cancers</td>
<td>Telomerase inhibition</td>
<td>Not evaluated</td>
</tr>
<tr>
<td>Kinases</td>
<td>HDAC inhibitors</td>
<td>Melanoma, nasopharyngeal and cervical cancers</td>
<td>Telomerase inhibition</td>
<td>Not evaluated</td>
</tr>
<tr>
<td>Kinases</td>
<td>PCK inhibitors</td>
<td></td>
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</tr>
</tbody>
</table>

They include Myc (Wang et al. 1998), Sp1 (Kyo et al. 2000), the estrogen and androgen receptors (Misiti et al. 2000, Bosland 2003), E2F-1 (Crowe & Nguyen 2001), WT-1 (Oh et al. 1999), nuclear factor-kB (Yin et al. 2000) and MZF-2 (Fujimoto et al. 2000). Among them, Myc has been studied in the greatest detail.

The c-Myc oncoprotein has been found to be overexpressed in a wide variety of tumor types including prostate cancers (Dalla-Favera et al. 1982, Cole 1986, Lee et al. 1996). The importance of c-Myc in tumorigenesis has been demonstrated in various experimental studies. However, the mechanisms by which c-Myc participates in tumorigenesis are not yet fully understood. Identification of c-Myc targets, which regulate cell growth, both positively and negatively, may in part explain the role of c-Myc in controlling cell proliferation. The c-Myc oncogene has recently been implicated in the regulation of telomerase through transcriptional activation of telomerase reverse transcriptase, the limiting component for telomerase activity (Weinrich et al. 1997, Bodnar et al. 1998, Vaziri & Benchimol 1998, Wang et al. 1998, Greenberg et al. 1999, Wu et al. 1999, Kyo et al. 2000). A link between Myc expression and telomerase activity has also been observed in tumors. In particular, hTERT expression correlates with Myc overexpression in prostate, neuroblastoma and cervical cancers (Hiyama et al. 1995b, Latil et al. 2000, Sagawa et al. 2001). In this context, we recently found that c-Myc-dependent telomerase activation plays a crucial role in melanoma tumorigenicity (Biroccio et al. 2002a). In particular, by using melanoma-derived clones expressing low levels of c-Myc, we demonstrated that the downregulation of c-Myc reduced cell proliferation rate, cloning efficiency and tumorigenicity and increased the apoptotic rate. Decreased tumorigenic potential correlated with reduced hTERT gene expression, telomerase activity and telomere shortening. Introduction of hTERT into these cells increased their proliferation rate and partially re-established their genetic potential, demonstrating that the reconstitution of high levels of telomerase activity reverses the low tumorigenicity due to low c-Myc expression. By using the same experimental model, we also found that the down-regulation of c-Myc oncoprotein decreased the intracellular glutathione content by reducing the expression and activity of γ-glutamyl-cysteine synthetase, the rate-limiting enzyme for glutathione biosynthesis (Biroccio et al. 2002b). The two independent c-Myc-mediated biological effects, telomerase dysfunction and oxidative stress, cooperated in inducing cellular crisis (Biroccio et al. 2003a). Concomitantly, we also demonstrated that c-Myc-dependent telomere dysfunction is involved in the increased sensitivity to anti-neoplastic drugs (Biroccio et al. 2003b).

In particular, we found that the downregulation of c-Myc increased the susceptibility to cisplatin and to the novel anti-cancer drug eteinascidin-743. Reconstitution of hTERT in the c-Myc low-expressing clones restored telomerase activity, reduced telomere dysfunction and decreased the sensitivity to both drugs, by enabling the cells to recover from drug-induced G2/M block and consequently protecting them from apoptosis.

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Guanine methylation/demethylation processes were also considered as modulating hTERT expression regulation, since the promoter of the hTERT gene has a dense CG-rich CpG island (Devereux et al. 1999, Dessain et al. 2000). A positive correlation was observed among hypermethylation of the hTERT promoter, hTERT mRNA expression and telomerase activity (Guilleret et al. 2002). Furthermore, this correlation was confirmed in normal tissues where hypermethylation of the hTERT promoter was found exclusively in hTERT-expressing telomerase-positive samples and was absent in telomerase-negative samples (Guilleret et al. 2002). Moreover, the same group recently demonstrated that demethylation of the hTERT gene promoter by 5-azacytidine reduced hTERT expression, telomerase activity and shortened telomeres (Guilleret & Benhattar 2003).

The inhibitory effect of 5-azacytidine on telomerase activity has also been demonstrated on prostate tumors (Kitagawa et al. 2000). In particular, treatment of prostate cancer cells with 5-azacytidine significantly reduced telomerase activity and inhibited cell growth. Inhibition of telomerase activity was accompanied by transcriptional downregulation of hTERT mRNA expression. Further analysis seems to indicate that p16 and c-Myc may play a key role in the 5-azacytidine-mediated transcriptional repression of hTERT (Kitagawa et al. 2000).

Histone deacetylase inhibitors
Emerging evidence suggests that reversible acetylation/deacetylation of nucleosomal histones and the resultant changes in the chromatin structure are important processes in gene transcription regulation. Acetylation of histones leads to chromatin decondensation, increasing the accessibility for RNA polymerase complexes. Chromatin remodeling is a dynamic process catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC). Transcription factors, such as Mad, can repress gene expression by recruiting HDACs to specific sites in the gene promoters (Hassig et al. 1997, Laherty et al. 1997). Since hTERT promoter contains Mad-binding sites, histone acetylation may be involved in the transcriptional regulation of hTERT. However, the role of histone acetylation in the regulation of hTERT expression is controversial. It has been demonstrated that tricostatin A (TSA), a potent HDAC inhibitor, was able to reduce telomerase activity in liver and prostate cancers by decreasing the levels of the hTERT mRNA (Suenaga et al. 2002). On the contrary, other groups have shown that TSA induced a significant activation of hTERT mRNA and telomerase activity in telomerase-negative cells (Cong & Bacchetti 2000) and attenuated the repression of hTERT during HL60 cell differentiation (Xu et al. 2001).

Kinase inhibitors
Phosphorylation of hTERT protein is one of the mechanisms of hTERT activation. Some protein kinases, such as PKC, involved in several cancer-associated signal transduction pathways, appear to enhance telomerase activity (Li et al. 1998, Kang et al. 1999). On the contrary, protein phosphatase 2A (PP2A) inhibits telomerase activity (Li et al. 1997). On the basis of these findings, kinase-modulating drugs have been used.

In particular, two PKC inhibitors, bis-indolylmaleimide I and H-7, were found to produce a significant inhibition of telomerase activity in nasopharyngeal carcinoma cells (Ku et al. 1997) and during T cell activation (Bodnar et al. 1996). Similar results were obtained in cervical cancer cell lines treated with bis-indolylmaleimide I (Kim et al. 2001). The decrease in telomerase activity by PKC inhibition seems to be strictly related to direct or indirect phosphorylation of telomerase proteins, including hTERT. Other PKC inhibitors, such as staurosporine and sphingosine, were less active in decreasing telomerase activity, and other inhibitors, such as quercetin, H-89 and herbimycin A, did not significantly block telomerase activity.

Treatment of human melanoma cells with PP2A decreased telomerase activity, while okadaic acid, an inhibitor of PP2A, stimulated both hTERT phosphorylation and telomerase activity (Kang et al. 1999, Yu et al. 2001).

Approaches targeting telomerase-positive cells
On the basis that telomerase is typically absent from most normal human cells, but is expressed in nearly all human cancer cells (Kim et al. 1994, Broccoli et al. 1995, Hiyama et al. 1995a), two strategies aimed at targeting telomerase-positive cells have been investigated.

Gene therapy using hTERT promoter
The promoter of hTERT was used to restrict the expression of therapeutic genes to telomerase-positive cells. In fact, because the hTERT gene is regulated at the transcription level, only tumor cells expressing hTERT would activate the promoter. Much effort has been focused on transferring apoptotic-related genes downstream from the hTERT proximal promoter (from −1 to about 200 to −400). It has been reported that transduction of apoptotic genes such as caspase 6.8 (Komata et al. 2001a, 2002), Fas-associated protein with death domain (FADD) (Koga et al. 2001b, Komata 2001b) and bax (Gu et al. 2000, 2002) using the hTERT promoter induced apoptosis in hTERT-positive tumor cells, but not in normal cells or in tumor cells possessing alternative
mechanisms to lengthening telomeres. Moreover, using these constructs, the growth of tumors in nude mice was significantly suppressed and toxicity prevented.

Interesting results have been obtained using telomerase-specific oncolytic virus (Wirth et al. 2003, Kawashima et al. 2004). The adenoviral vector was able to infect both normal and tumor cells, but only the cells expressing telomerase activity transcriptionally activated the hTERT promoter, permitting viral replication and eventually lysis of cells. Wirth et al. (2003) used a telomerase-dependent conditionally replicating adenovirus (Ad) demonstrating adenoviral replication of hTERT-Ad in all investigated telomerase-positive tumor cell lines but not in telomerase-negative primary human hepatocytes. The capability of hTERT-Ad to induce cytopathic effects in tumor cells was comparable with that of the wild-type adenovirus and significantly higher compared with the oncolytic virus ONYX-015, regardless of the p53 status of the tumor cells, both in vitro and in vivo. Moreover, very recently the effect of a tumor-specific replication competent adenoviral strategy (hTERTp-TRAD) in human cancer cells has been investigated (Kawashima et al. 2004). TRAD induced E1A and E1B adenoviral gene expression in human cancer cells, but not in normal cells such as human fibroblasts. TRAD replicated efficiently and induced marked cell killing in a panel of human cancer cell lines, whereas replication as well as cytotoxicity was highly attenuated in normal human fibroblasts lacking telomerase activity. In mice carrying human lung tumor xenografts, intra-tumoral injection of TRAD resulted in a significant inhibition of tumor growth. No evidence of TRAD was identified in tissues outside of the tumors, despite the presence of TRAD in the circulation. Notwithstanding the good results obtained with the intra-tumoral injection of TRAD, systemic hTERTp-TRAD could have side-effects on proliferating cells expressing telomerase activity.

**hTERT immunotherapy**

The immunological properties of hTERT that have been discovered suggest that the enzyme is also an attractive target for novel immunotherapies against cancer. To date, there are several reports demonstrating anti-hTERT cytotoxic T lymphocytes (CTL) responses in cancer. In particular, CD8+ CTL specific for hTERT peptides and restricted to major histocompatibility complex (MHC) HLA-A2 lysed telomerase-positive tumors of different histotypes (Vonderheide et al. 1999, Minev et al. 2000, Arai et al. 2001). Interestingly, no CTL effect was found in telomerase-negative cells, cells negative for the human leucocyte antigen-A2 or telomerase-positive CD34+ hematopoietic cells (Vonderheide et al. 1999, Minev et al. 2000). Furthermore, induction of CTL responses and tumor immunity against unrelated tumors using TERT RNA-transfected dendritic cells has been demonstrated (Nair et al. 2000). On the basis of these promising results, a phase I clinical trial was performed to evaluate the clinical and immunological impact of vaccinating advanced cancer patients with the HLA-A2-restricted hTERT 1540 peptide (Vonderheide et al. 2004). hTERT-specific T lymphocytes were induced in four of seven patients with advanced breast or prostate carcinoma after vaccination with dendritic cells pulsed with hTERT peptide. Tetramer-guided high-speed sorting and polyclonal expansion achieved highly enriched populations of hTERT-specific cells that killed tumor cells in an MHC-restricted fashion. Despite concerns about telomerase activity in rare normal cells, no significant toxicity was observed. Partial tumor regression in one patient was associated with the induction of CD8+ tumor-infiltrating lymphocytes.

**Conclusions and perspectives**

Different approaches that directly or indirectly target telomerase have been developed in cancer cells.

According to the initial paradigm for telomerase inhibitors, telomerase inhibitors should initially decrease telomerase activity without affecting the growth rate. Decreased proliferation should only be observed when the telomeres reach a critically short length. Therefore, the delay necessary for the anti-proliferative effect should depend on the initial telomere length. In agreement with this assumption, preclinical studies demonstrated that most telomerase inhibitors are effective on a wide variety of malignancies possessing short telomeres. The lag phase between the time at which telomerase is inhibited and the time at which telomeres shortened sufficiently to produce detrimental effects on cancer cells is evidently a serious obstacle for the application of anti-telomerase strategy, especially in tumor cells with long telomeres. Moreover, the long time exposure to anti-telomerase agents can lead to the development of resistant tumor cells through overexpression of telomerase activity or reactivation of alternative telomere-lengthening mechanisms. One way to overcome this limitation is by combining telomerase inhibition with DNA-damaging chemotherapeutic drugs. Different groups, including ours, have found an enhanced sensitivity of telomerase-inhibited cancer cells to common chemotherapeutic drugs, suggesting that combining anti-telomerase strategies with chemotherapy could efficiently improve the response of tumors.

Recent findings demonstrated that changes in telomere uncapping versus capping status might be as important as actual telomere length in determining cell survival or death. Great effort has therefore been focused
on the development of new agents that directly induce telomere dysfunction. The most important advantage in the use of telomere interacting molecules, rather than telomerase inhibitors, is the possibility of obtaining an anti-cancer effect in both telomerase-positive and telomerase-negative tumor cells with long telomeres. However, it must be pointed out that an anti-telomere approach might be more risky, as it may more readily affect telomerase-negative somatic cells.

To date, the most promising strategy for telomerase inhibition seems to be the disruption of telomere capping by telomerase. It has been reported that telomerase, taking part in telomere capping, can regulate cell survival independent of telomerase activity. This approach might even work in tumors with long telomeres, maintaining the specificity against tumor versus normal cells. Thus, a better understanding of the functions of telomerase, as distinct from its telomere-elongating role, should give rise to a completely new generation of telomerase inhibition approaches.

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