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T-oligo as an anticancer agent in colorectal cancer

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ABSTRACT

In the United States, there will be an estimated 96,830 new cases of colorectal cancer (CRC) and 50,310 deaths in 2014. CRC is often detected at late stages of the disease, at which point there is no effective chemotherapy. Thus, there is an urgent need for effective novel therapies that have minimal effects on normal cells. T-oligo, an oligonucleotide homologous to the 3′-telomere overhang, induces potent DNA damage responses in multiple malignant cell types, however, its efficacy in CRC has not been studied. This is the first investigation demonstrating T-oligo-induced anticancer effects in two CRC cell lines, HT-29 and LoVo, which are highly resistant to conventional chemotherapies. In this investigation, we show that T-oligo may mediate its DNA damage responses through the p53/p73 pathway, thereby inhibiting cellular proliferation and inducing apoptosis or senescence. Additionally, upregulation of downstream DNA damage response proteins, including E2F1, p53 or p73, was observed. In LoVo cells, T-oligo induced senescence, decreased clonogenicity, and increased expression of senescence associated proteins p21, p27, and p53. In addition, downregulation of POT1 and TRF2, two components of the shelterin protein complex which protects telomeric ends, was observed. Moreover, we studied the antiproliferative effects of T-oligo in combination with an EGFR tyrosine kinase inhibitor, Gefitinib, which resulted in an additive inhibitory effect on cellular proliferation. Collectively, these data provide evidence that T-oligo alone, or in combination with other molecularly targeted therapies, has potential as an anti-cancer agent in CRC.

1. Introduction

Telomeres, the protective structures found at the ends of chromosomes, contain 3′ overhangs composed of TTAGGG tandem DNA repeats that form telomeric loop structures (T-loop) that maintain telomere integrity [1,2]. Following successive rounds of DNA replication, telomere length is progressively reduced [3]. When substantial telomere attrition occurs, telomeres become unstable, thereby triggering apoptosis or senescence and limiting the proliferative potential of normal cells [4,5]. However, in cells that inappropriately express telomerase, telomeres are stabilized, which allows for continued proliferation and promotes malignant transformation [5]. Since 90% of tumor types have been shown to have elevated levels of telomerase [6], the induction of DNA damage responses (DDRs) following telomeric DNA loss may be an important and near-universal tumor suppressor mechanism [5]. Hence, strategies aimed at disrupting telomere ends or mimicking telomere exposure are novel and attractive approaches for treating a broad range of cancer types [5,7].

Administration of an oligonucleotide homologous to the 3′ telomere overhang, T-oligo, induces potent DDRs in numerous malignant cell types, including cell-cycle arrest and cancer cell-type specific apoptosis or senescence [8–13], while sparing their non-transformed counterparts [13,14]. T-oligo accumulates in the nucleus [9,14] and induces DDRs characterized by phosphorylation of H2AX, ATM, cdk2, and p53 [9–11,14,15]. Interestingly, the cytostatic and cytotoxic effects of T-oligo are comparable to those following telomere dysfunction induced by ectopic expression of a dominant negative TRF2, which results in senescence or apoptosis and is also mediated in part by ATM and p53 [1]. However, sequences complementary or unrelated to T-oligo have no reported cytotoxic effects [9–11]. Hence, T-oligo is thought to mimic damaged telomeric DNA [16], and thus has potential as a novel anticancer therapeutic in multiple cancer types [17].

Since the efficacy of T-oligo has not been investigated in CRC, we explored T-oligo's mechanism of action and therapeutic

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potential in HT-29 and LoVo CRC cell lines, which are highly tumorigenic [18,19]. We demonstrate T-oligo-induced senescence, inhibition of cell growth, cell death, and differentiation, and further characterize important downstream signaling proteins involved in these responses. We show for the first time that T-oligo downregulates shelterin complex proteins TRF2 and POT1, which are integral for telomere stability [2]. T-oligo treatment also upregulated differentiation markers villin and mucin, which are lost in poorly differentiated CRC cells [20,21]. In addition, we studied the anticancer effects of T-oligo combined with an EGFR tyrosine kinase inhibitor (EGFR-TKI), Gefitinib, since EGFR inhibitors being used as first-line treatments for CRC have limited efficacy [22]. This study establishes the potential therapeutic efficacy of T-oligo in CRC, and extends our understanding of its underlying mechanisms of action.

2. Materials and methods

2.1. Oligonucleotides and intracellular uptake of FITC-T-oligo

An oligonucleotide homologous to the 3′ telomere overhang sequence, T-oligo (pGTTAGGGTTAG), FITC-labeled T-oligo, and its complementary sequence C-oligo, (pCTAACCCTAAC), were obtained from Midland Certified Reagent Company (Midland, TX). The uptake of FITC-T-oligo was performed as described previously [23].

2.2. Cell culture

LoVo (Cat No. CCL-229) and HT-29 (Cat No. HTB-38) CRC cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HT-29 cells were maintained at 37 °C in McCoy's 5a Medium Modified (Cat No. 30-2007 ATCC, Rockville, MD) with 10% fetal bovine serum (Cat No. S11150, Atlanta Biologicals, Lawrenceville, GA) and 1% (v/v) antibiotic/antimycotic (Cat No. 15240 Invitrogen, Grand Island, NY), according to ATCC's instructions. LoVo cells were grown in F-12K Media (Cat No. 30-2004, ATCC, Rockville, MD) with 10% fetal bovine serum and 1% (v/v) antibiotic–antimycotic.

2.3. Immunoblotting and antibodies

HT-29 and LoVo cells were cultured as described above and immunoblotting was performed as described previously [24]. The following antibodies were used: E2F1 (Cat No. SC251, Santa Cruz Biotechnology, Santa Cruz, CA), antiphospho-histone H2AX (Cat No. 05–636), p53 (Cat No. OP43) and p73 (Cat No. PC385) (EMD Millipore, Billerica, MA), Nbs1 (Cat No. 3002) and phospho-Nbs1 (Cat No. 3001) (Cell Signaling Technology, Beverly, MA), anti-Cip1/WAF1 (Cat No. 610233) and anti-p27Kip1 (Cat No. 610241) (BD Transduction Laboratories, San Diego CA), TRF2 (Cat No. NB110-57130) and POT1 (Cat No. NB500-176) (Novus Biologicals, Littleton, CO), and beta-actin (Cat No. A5316, Sigma–Aldrich, St. Louis, MO). For immunofluorescence, the following antibodies were used: Anti-mouse Alexa fluor 488 secondary antibody (A-11001) and Alexa fluor 594 secondary antibody (Cat No. S11227) (Life Technologies, Grand Island, NY), and villin (Cat No. 610359, BD biosciences, Franklin lakes, NJ). All antibodies were used according to manufacturer instructions.

2.4. Cell cycle analysis

Cells were cultured and plated as described above and treated as described previously [16]. Cells were collected by trypsinization, fixed in 35% ethanol/65% DMEM, treated with RNase A and stained with propidium iodide (PI) (Cat No. 81845: Sigma–Aldrich; St. Louis, MO). PI staining and cell cycle analysis was performed as described previously [8,11]. All experiments were performed in triplicate.

2.5. Cell death and inhibition of cell growth

HT-29 cells were grown and treated for 24–72 h and stained with PI as described earlier. Cell death was also determined as described previously [11]. For determining cellular proliferation, cells were trypsinized and counted with a coulter counter. Prior to all treatment conditions, cell viability was found to be more than 95% as determined by trypan blue exclusion.

2.6. Senescence and clonogenicity

Senescence in LoVo cells was determined as described previously [25]. For determination of clonogenicity, LoVo cells were plated at 25,000 cells/60 mm dish and treated as described above for 1 week. Cells were harvested with trypsin/EDTA, plated at 3000 cells/60 mm dish, and incubated for eight days in growth medium. Cells were then fixed in 100% ethanol and stained with 1% methylene blue in PBS for 10 min. Visible colonies were counted using Kodak ROI analysis. All experiments were performed in triplicate. Paired Student's *t*-test was used to evaluate the differences between groups. Significance was established at $\alpha = 0.05$.

2.7. Gamma-H2AX and TRF2 immunofluorescence

10,000 HT-29 or LoVo cells were plated on glass chamber slides (Lab-TeK, Scotts Valley, CA) and allowed to adhere for 24 h in serum-free medium. Cells were then treated with T-oligo for 24 h and fixed with 95% methanol and 5% acetic acid for 5 min, and blocked with blocking buffer (3% BSA/TBS) for 30 min. Cells were treated with antiphospho-Histone H2AX antibody in blocking buffer for 1 h, washed three times with TBS, and then anti-rabbit Alexa fluor 488 (green) secondary antibody in blocking buffer was added and incubated in the dark for 1 h. Cells were washed five times, mounted and observed under microscope. Hoechst dye (Cat No. H1399, Life Technologies, Grand Island, NY) was used for nuclear staining (blue). For TRF2 immunofluorescence, cells were plated and treated as above and then fixed with 10% formalin for 30 min at room temperature. The formalin was then removed and ice cold methanol was added for 10 min. Methanol was then removed and cells were washed three times with PBS for 10 min. The blocking was performed with 10% normal goat serum for 1 h at room temperature. Anti-TRF2 primary antibody was added for 2 h, washed three times with PBS. Alexa fluor 594 secondary antibody was then added and incubated for 1 h, and then washed three times with PBS. Cells were then incubated for 10 min in Hoechst dye and washed with PBS three times. Immunofluorescence was observed using an Olympus "live cell" DSU Spinning Disk inverted confocal fluorescent microscope. Total fluorescence measurements were performed with Image J software.

2.8. Detection of mucin

Cells were treated as described above, fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% triton X-100 for 2 min and then treated with 0.5% periodic acid for 5 min, washed with distilled water and placed in Schiff reagent (Electron microscopy sciences, Hatfield, PA) for 15 min. Images were taken using an Olympus BH-2 microscope.

2.9. WST-1 cell viability assay

Cells were plated at 5000 cells/well in 96-well plates and incubated for 24 h. Cells were treated with 20 μM T-oligo, 2.5 μM Gefitinib (Cat No. G-4408, LC Laboratories, Woburn, MA) or 20 μM T-oligo combined with 2.5 μM Gefitinib for 96 h. Each experiment was performed in replicates of six for each treatment condition and repeated three times. At the indicated time points after plating, cell viability was determined using the cell proliferation reagent WST-1 (Cat No. 05015944001, Roche Applied Science, Indianapolis, IN) by measuring absorbance at 450 and 540 nm on an EL 800 plate reader (Biorad, Hercules, CA) according to the manufacturer's instructions. Absorbance differences (450–540 nm) were calculated as means ± SD and expressed as a percentage of untreated controls.

3. Results

3.1. Exposure to T-oligo induces phosphorylation of H2AX and Nbs1, as well as S-phase arrest

At sites of DNA damage, H2AX initiates foci formation and recruits DNA repair proteins such as Nbs1 [26], a regulator of cell cycle progression [27]. Since T-oligo is known to induce cell cycle arrest [10,11], we were interested in examining whether it modulates H2AX, Nbs1, and cell cycle progression in CRC cells. Initially, FACS analysis demonstrated a log-fold increase in cellular uptake of T-oligo compared to cells treated with diluent (Fig. 1A). After treatment with T-oligo, gamma-H2AX foci formation was observed in CRC cells at 24 h (Fig. 1B). T-oligo upregulated gamma-H2AX in HT-29 (6.2-fold) and LoVo (3-fold) cells at 24 h (Fig. 1B). In HT 29 cells, treatment with T-oligo for 24 h more than doubled the percentage of cells in S-phase (50.4 ± 0.2%) compared to cells treated with either diluent (20.8 ± 2%) or C-oligo (20.9 ± 1.5%), respectively (Fig. 1C). Similar results were obtained in LoVo cells (data not shown). In HT-29 cells, we observed upregulation of Nbs1 phosphorylation (3-fold) at 12 h (Fig. 1D).

3.2. T-oligo induces cell death, inhibits proliferation, and upregulates expression of DDR proteins

T-oligo induced a 2.5- and 3.0-fold increase in cell death in HT-29 cells compared to treatment with diluent or C-oligo at 48 and 72 h, respectively ($p < 0.01$, Fig. 2A). Moreover, T-oligo inhibited cellular proliferation in HT-29 (58%, $p < 0.03$) and LoVo (50%, $p < 0.03$) cells at 96 and 72 h, respectively, compared to controls (Fig. 2B). To further investigate proteins known to mediate DDRs, we studied p53, p73, and their downstream target, p21. E2F1, a transcription factor for p53 and p73, was upregulated in LoVo (2.5-fold) and HT-29 (11-fold) cells (Fig. 2C, D). In the p53-positive LoVo cells, we found that treatment with T-oligo increased expression of p53 (2.0-fold) at 24 h, as well as p21 at both 24 and 48 h (2.0- and 3.0-fold, respectively) (Fig. 2C). In addition, p73 was strongly upregulated (27-fold) at 48 h, suggesting that T-oligo-induced cell death may be mediated by p73 in the absence of p53 (Fig. 2D).

3.3. T-oligo induces senescence, upregulates senescence markers and decreases clonogenicity in LoVo cells

Since both telomere shortening and exposure elicit senescence [4], we investigated whether T-oligo could mimic this signal and induce senescence in CRC cells. 44.8% of cells treated with T-oligo were senescence associated beta-galactosidase (SA beta-gal) positive compared to only 3.9% and 4.5% of cells treated with diluent and C-oligo, respectively ($p < 0.001$, Fig. 3A). T-oligo-treated cells were also larger in size, which is characteristic of cells undergoing senescence (Fig. 3A). Furthermore, T-oligo increased expression of senescent markers p53 (2.0-fold), p27 (1.8-fold), and p21 (2.0-fold) (Fig. 3B). In addition, senescent LoVo cells showed a 43% reduction in clonogenicity, which is a reliable measure of therapeutic response to chemotherapeutic agents ($p < 0.01$, Fig. 3C) [28].

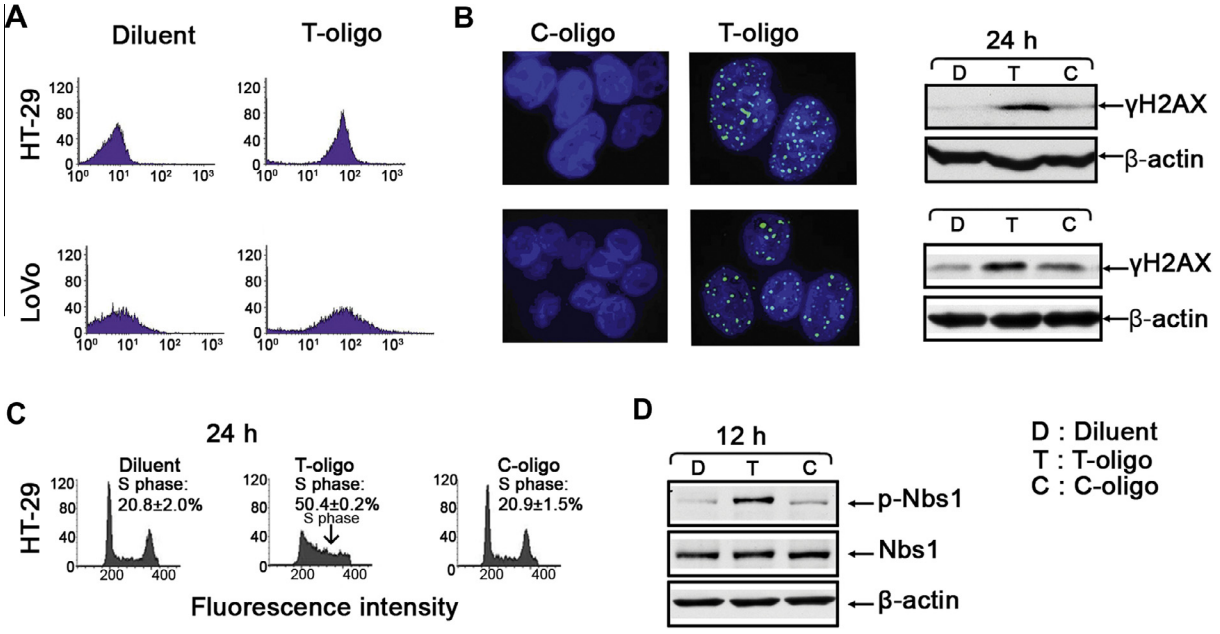

Fig. 1. Cellular uptake of T-oligo and its effect on gamma-H2AX, induction of S-phase arrest, and phosphorylation of Nbs1. (A) Log fold increase in cellular uptake of T-oligo. (B) Formation of gamma-H2AX foci (green) in both HT-29 and LoVo cells after T-oligo treatment. Gamma-H2AX was upregulated in HT-29 (6.2-fold) and LoVo cells (3-fold). (C) In cells treated with T-oligo, 50.4 ± 0.2% of cells were in S-phase at 24 h. In cells treated with diluent or C-oligo, 20.8 ± 2% and 20.9 ± 1.5% of cells were in S-phase at 24 h (D). Upregulation of Nbs1 phosphorylation (4.3-fold). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

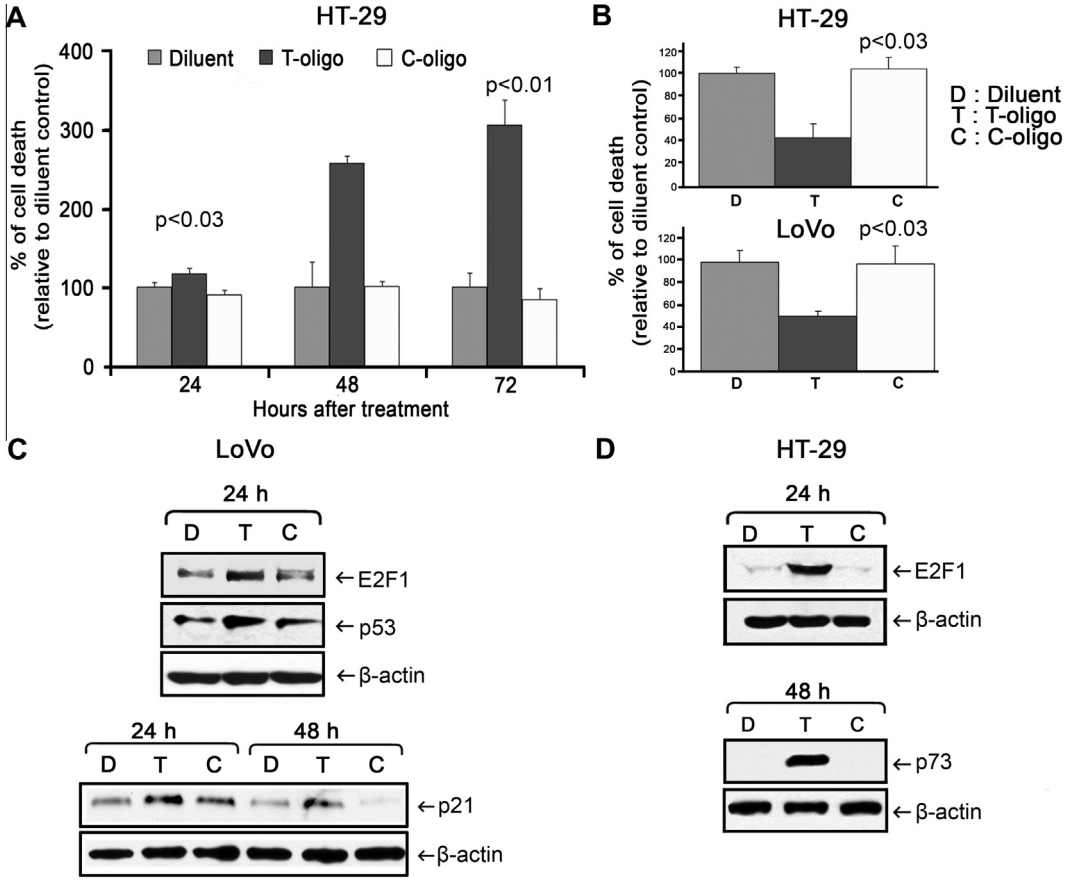

Fig. 2. Treatment with T-oligo induces cell death, inhibits proliferation, and upregulates expression of DNA damage response proteins. (A) A 2.5–3.0-fold increase in cell death was seen only with T-oligo treatment. (B) T-oligo treatment decreased cell numbers in HT-29 (58%), and LoVo (50%) cells. (C) Treatment with T-oligo increased the expression of E2F1 (2.5-fold), p53 (2.0-fold) at 24 h, and p21 (2.0–3.0-fold) at 24–48 h in LoVo cells. (D) T-oligo-induced upregulation of E2F1 (11-fold, 24 h) and p73 (27-fold, 48 h) in HT-29 cells.

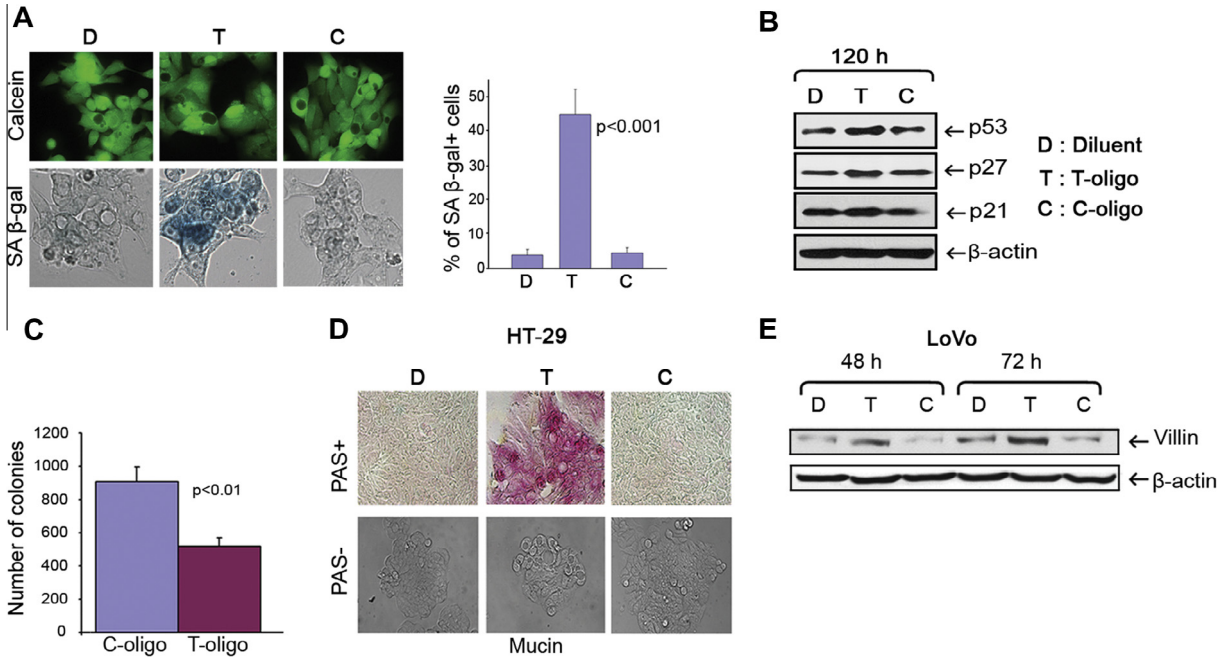

Fig. 3. T-oligo exposure induces senescence, decreases clonogenicity, and upregulates differentation markers in CRC cells. (A) Cells treated with T-oligo exhibited senescent phenotypes. 44.8% of T-oligo treated cells were SA beta-gal positive, compared to less than 5% in controls. (B) LoVo cells treated with T-oligo resulted in increased expression of p53 (2.0-fold), p27 (1.8-fold), and p21 (2.0-fold). (C) T-oligo treated cells exhibited a 43% reduction in visible colonies. (D) Upregulation of mucin at 72 h in HT-29 cells. (E) Upregulation of villin (1.5–3.0-fold) in LoVo cells.

3.4. T-oligo upregulates expression of differentiation markers mucin and villin

Previous reports by our lab have demonstrated that T-oligo increases the expression of differentiation markers in melanoma [16]. Thus, we investigated T-oligo-induced differentiation in CRC cells. Mucins were strongly upregulated in HT-29 cells after T-oligo treatment, compared to C-oligo or diluent at 72 h (Fig. 3D). T-oligo treatment also upregulated villin at 48 h (1.5-fold) and 72 h (3.0-fold) in LoVo cells (Fig. 3E).

3.5. T-oligo downregulates telomere-associated proteins POT1 and TRF2

Functional levels of TRF2 and POT1 are essential for proper telomere maintenance and stability of the T-loop [2,29]. We demonstrated that T-oligo treatment downregulated TRF2 in LoVo (2.4-fold, $p < 0.01$) and HT-29 (1.8-fold, $p < 0.02$) cells compared to C-oligo treatment (Fig. 4A), as assessed by immunfluorescence. Additionally, T-oligo treatment downregulated POT1 in both HT-29 (2-fold) and LoVo (3-fold) cells at 48 and 72 h, respectively (Fig. 4B) as seen by immunoblotting.

3.6. T-oligo combined with Gefitinib induces additive inhibitory effects on cellular proliferation

To investigate the therapeutic efficacy of T-oligo in combination with Gefitinib, HT-29 cells were treated with T-oligo, Gefitinib, or T-oligo plus Gefitinib, at submaximal but effective inhibitory doses, and cellular proliferation was measured by WST-1 assay. Percent inhibition of HT-29 cell proliferation was 9.5 ± 4.4% for Gefitinib, 25.0 ± 5.0% for T-oligo, and 34.9 ± 4.5% for the combination of Gefitinib and T-oligo ($p < 0.01$, Fig. 4C).

4. Discussion

Treatment of metastatic CRC is challenging, as currently used molecularly targeted therapies are mostly ineffective as single agents, and have accompanying adverse side-effects when used in combination [30]. T-oligo, an oligonucleotide homologous to the telomere overhang, elicits numerous anticancer responses in a variety of cancer types [11–14,25,31]. In this investigation, we demonstrate that T-oligo induces a diverse range of anticancer effects in CRC cells, such as S-phase arrest, growth inhibition, cell death or senescence, and differentiation. We also observed downregulation of telomere associated proteins TRF2 and POT1. Finally, we demonstrated that T-oligo induces additive antiproliferative effects when combined with an EGFR-TKI. This study establishes for the first time that T-oligo could be an effective therapeutic agent for CRC.

H2AX recruits DNA repair proteins such as Nbs1 that co-localize with gamma-H2AX foci at sites of DNA damage [26]. Nbs1 is required for the activation of S-phase arrest [8,27] and previous studies have shown that mutant Nbs1 significantly abrogates T-oligo-induced S-phase arrest [32]. We report that CRC cells exposed to T-oligo experience an S-phase arrest, which likely contributes to their inhibition of cellular proliferation [25]. We also found increased transcription of p53/p73 in CRC cells, and our earlier studies indicate that p53 is a key component in T-oligo's mechanism of action. However, T-oligo's cytostatic effects are not dependent solely on a functional p53 [12,13], as we have earlier demonstrated p73 mediated T-oligo anticancer responses in p53-deficient melanoma cells [16,25]. In this study we further demonstrate that p73 can mediate DDRs in HT-29 cells, which have a nonfunctional p53. While cell death was observed in HT-29 cells after T-oligo treatment, LoVo cells did not exhibit significant T-oligo-induced cell death, which may require p73. Formation of gamma-H2AX foci and the upregulation of the above proteins suggest that they may have a role in T-oligo-induced DDRs in CRC cells.

Induced telomere dysfunction, as achieved via ectopic expression of a dominant negative TRF2, initiates telomere loss and rapidly activates p53-dependent senescence [33]. Since T-oligo is presumed to destabilize telomeres [16] without decreasing their length [8,10,15], we examined possible T-oligo mediated senescence in CRC cells. Upregulation of senescent markers and

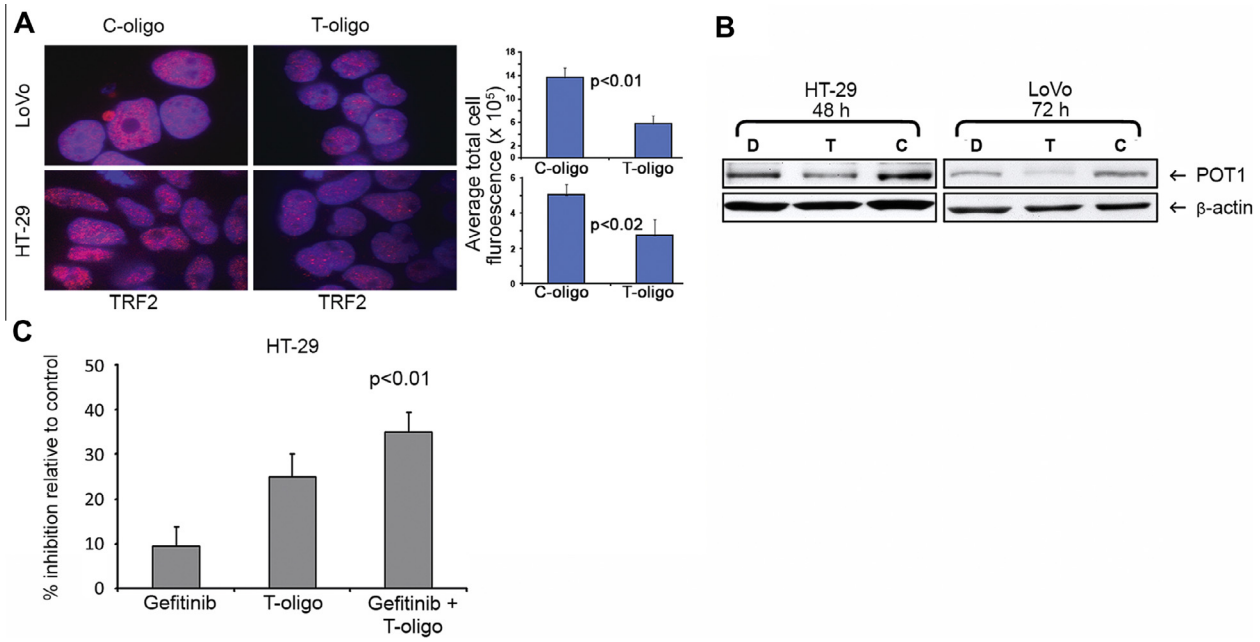

Fig. 4. T-oligo downregulates telomere associated proteins POT1 and TRF2, and induces additive antiproliferative effects when combined with an EGFR-TKI. (A) Downregulation of TRF2 in LoVo (2.4-fold) and HT-29 (1.8-fold) cells. (B) Downregulation of POT1 in HT-29 cells (2.0-fold) at 48 h and in LoVo cells (3.0-fold) at 72 h. (C) The antiproliferative effects of Gefitinib+T-oligo were additive. Percent growth inhibition in HT-29 cells treated with Gefitinib (9.5 ± 4.4%) T-oligo (25.0 ± 5.0%), and Gefitinib+T-oligo (34.9 ± 4.5%) ($p < 0.01$).

SA-beta galactosidase staining was observed in LoVo cells. In contrast, T-oligo failed to induce significant senescent in HT-29 treated cells (data not shown), which could suggest that T-oligo-induced senescence requires functional p53 and/or downstream targets such as p27, which are upregulated in senescent LoVo cells. Upregulation of senescent markers was observed even after 120 h of continuous treatment, indicating that their sustained upregulation was perhaps mediating senescence.

The colonic epithelium is protected and lubricated by mucins, which are highly glycosylated proteins [34]. Mucin is a marker of differentiation, and the most abundantly secreted mucins are downregulated in intestinal carcinomas, resulting in increased tumorigenicity and metastasis [34]. Villin, another differentiation marker, is also frequently lost in poorly differentiated CRCs [20]. Villin is expressed in the intestinal epithelium and regulates the actin cytoskeleton, as it can cap, sever, and bundle actin filaments. Upregulation of these markers as observed in this study indicates that T-oligo might inhibit cellular proliferation and invasion by promoting differentiation [21].

Shelterin proteins POT1 and TRF2 play important roles in the maintenance of telomere integrity and length [29]. POT1, a single-stranded telomere binding protein, plays a key role in telomere end protection [5]. Overexpression of TRF2 inhibits the activation of ATM, thereby suppressing ATM-dependent DDRs such as apoptosis and cell cycle arrest [35]. In general, deletion of these proteins results in rapid telomere loss and ultimately cell death [36]. Previous studies have shown that T-oligo does not induce telomere loss [8,10,15], however, recent investigations by our group suggest that T-oligo modulates expression levels of shelterin proteins, thereby altering telomere structure [16]. Thus, TRF2 and POT1 downregulation may cause telomere instability, which could promote activation of downstream mediators that induce DDRs such as cell death and senescence. We suggest that TRF2 and POT1 are implicated in T-oligo-mediated DDRs in CRC cells, and their resulting downregulation may promote telomere dysfunction.

Since our results demonstrate that a combination of T-oligo and an EGFR-TKI induced additive inhibition of cellular proliferation, compared to either individual agent alone, ongoing studies in our laboratory are aimed toward improving this and other combinatorial approaches. These proposed combinations include other EGFR inhibitors and telomere-based therapies, such as telomerase inhibitors and G-quadruplex stabilizers, which may help develop strategies to further increase their efficacies and bring them closer to clinical fruition. Future investigations will include studies using *in vivo* models, such as tumor xenografts and ApcMin mice, an animal model currently used to study CRC [37], to assess the efficacy of T-oligo in blocking tumorigenesis.

Disclosure

The authors declare no competing financial interests exist.

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