Xrcc3 and Nbs1 Are Required for the Production of Extrachromosomal Telomeric Circles in Human Alternative Lengthening of Telomere Cells

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Abstract

The maintenance of telomere length is essential for the indefinite proliferation of cancer cells. This is most often achieved by the activation of telomerase; however, a substantial number of cancers lack detectable telomerase activity and are classified as using an alternative lengthening of telomeres (ALT) pathway. We showed recently that ALT cells have a high level of extrachromosomal telomeric circles (t circles) that may be a specific marker of the ALT phenotype. The mechanism underlying t circle production and the requirement of t circles in ALT remain unclear. Understanding the specific requirements of ALT is key to developing diagnostic tools and therapies that target this pathway and is critical for the treatment of cancers in which ALT is prevalent, including cancers of neuroepithelial and mesenchymal origin. In this study, we used short hairpin RNAs directed at either Xrcc3 or Nbs1, two proteins involved in the homologous recombination pathway, to determine the role of these proteins in t circle production and the requirement of t circles in maintaining the ALT pathway. We show that Xrcc3 and Nbs1 are indeed required for the production of t circles in human ALT. However, these cells continue to proliferate in the absence of t circles, suggesting that they are not required for the survival of ALT cells. [Cancer Res 2007;67(4):1513–9]

Introduction

The ends of linear chromosomes consist of long tracts of repetitive DNA sequences that range from 5 to 15 kb in length and end with a single-stranded overhang of 150 to 200 nucleotides (1–3). Both the single-stranded overhang and the repetitive nature of telomeres are critical for maintaining telomere loops (t loops), the unique structure that results from the invasion of a single-stranded telomeric DNA overhang into the preceding duplex telomeric tract, therefore sequestering the chromosome ends away from DNA repair machinery (4–8).

Telomere length gradually declines over time partly due to the end replication problem and partly due to end processing events and this shortening acts as a mitotic clock that provides cells with a finite life span by signaling the onset of replicative senescence (9). As such, cancer cells must activate a telomere maintenance mechanism to establish immortalization. Telomerase is a reverse transcriptase enzyme that is capable of restoring eroded telomere sequences (10, 11). It can extend the proliferative life span of cells by catalyzing the addition of telomeric DNA onto the ends of telomeres using an internal RNA template complimentary to the telomere sequence (12, 13). Telomerase provides the most frequently activated telomere maintenance mechanism and is expressed in >85% of cancers (14). Therefore, much attention has been given to telomerase as a diagnostic marker and potential therapeutic target. However, there are many cancers particularly those of neuroepithelial and mesenchymal origin that have relatively high levels of tumors that do not use telomerase and instead use an alternative telomere maintenance mechanism [alternative lengthening of telomeres (ALT); refs. 14–16].

Human cancers that use this pathway are characterized by heterogeneous telomere lengths that range from 5 to 50 kb, which undergo rapid telomere elongation and shortening events (17, 18). In addition, ALT cells frequently contain a unique class of promyelocytic leukemia (PML) bodies called ALT-associated PML bodies (APB; ref. 19). These specialized bodies contain telomere binding proteins and telomeric DNA in addition to normal PML constituents that include a range of DNA repair proteins (19). ALT cells also contain circular extrachromosomal telomeric DNA [telomeric circles (t circles)] that can be separated from linear telomeric DNA by two-dimensional gel electrophoresis (20, 21).

Studies of patient-derived tumor samples suggest that ~50% of tumors are negative for both of these telomere maintenance mechanisms, suggesting that additional novel methods of telomere maintenance may exist (22, 23). A possible explanation for this is that some ALT cells may lack some of the classic features of the ALT phenotype, including the presence of APBs and t circles (24). Therefore, studies that use telomere length and APBs as a marker of ALT may actually underestimate the number of ALT-positive cells. Hence, more information is needed on the specific requirements of the ALT pathway and the necessity of these common ALT features in maintaining the ALT mechanism. Furthermore, telomerase has been proposed as a potential target for anticancer therapies; however, it remains possible that such therapies could lead to activation of the ALT pathway (25).

Our previous studies suggest that t circles are not present in telomerase-positive or normal cells, making them a key marker for the ALT phenotype (21). We have visually examined t circles in telomere-enriched fractions from ALT cells using electron microscopy (20, 21). The size of these t circles closely correlates with the size of the loop portion of the t loops, suggesting that t loops may be resolved into t circles in vivo (21). Although the mechanism underlying the production of t circles and their role in the ALT phenotype is unclear, it has been proposed that t circles result from homologous recombination events at telomeres (20, 21). This model is based on the close correlation between the size of t circles and the looped portion of t loops and the finding that the loss of
telomere length in cells expressing a mutant telomere binding protein (TRF2) is associated with the appearance of t circles (20, 21). Therefore, we have targeted two proteins known to be involved in the homologous recombination pathway, Nbs1 and Xrcc3, to determine the role of these proteins in t circle production in the ALT pathway and the requirement of t circles in maintaining the ALT phenotype.

Materials and Methods

Cell culture and proliferation rates. GM847, WI-VA13, and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), whereas Saos2 cells were maintained in McCoy's SA medium supplemented with 15% FBS. Cells were passaged twice weekly and maintained in log-phase growth. Isolation of individual cell clones was achieved by seeding 500 cells in a 15-cm tissue culture plate, and individual cell colonies were isolated by cylinder cloning and transferred to six-well plates and amplified into independent cultures. Cell growth was monitored by calculating the number of population doublings using the following equation population doublings = \[ \log (\text{cells counted} / \text{cells plated}) \] / 0.3 and plotted against time in days.

Short hairpin RNA sequences. Established nonspecific scrambled short hairpin RNA (shRNA) target sequences were purchased from OligoEngine, Inc. (SUPER Mamm-i-oligo set) and cloned into linearized pSUPER.retro construct according to the manufacturer's procedures (OligoEngine, Seattle, WA). Nbs1 small interfering RNA target sequences have been described previously in the literature and were used as target sequences for the synthesis of shRNA constructs suitable for ligation into the pSUPER.retro vector (26, 27). Nineteen nucleotide target sequences for Xrcc3 were previously in the literature and were used as target sequences for the synthesis of shRNA constructs suitable for ligation into the pSUPER.retro vector. (26, 27). Nineteen nucleotide target sequences for Xrcc3 were designed using Dharmaco software (Dharmacon Inc., Lafayette, CO). The Xrcc3 shRNA1 sequence GGATCTACTGGACCTGAAT and Xrcc3 shRNA2 sequence AGAAGGCTCCTCTTACT were used as targets for shRNA design.

Telomere length measurements and detection of t circles. Whole genomic DNA was extracted and incubated with RNase and restriction enzymes Ala1, Msp1, Rsa1, and HinI1 (New England Biolabs, Inc., Ipswich, MA) in Buffer 2 overnight at 37°C. Digested telomeric DNA was separated on a 1.0% pulse field gel electrophoresis (PFGE) gel for 25.5 h, 120° angle, and a 1 to 6 switch time at 1 volt/cm followed by visualization of DNA by ethidium bromide staining, excision of lanes, and running in a second dimension on a 1% PFGE for 9 h using a 1 to 6 switch time and 120° angle for 8.5 h (21). Both telomere length and t circle blots were denatured, dried, and neutralized before hybridization with a 32P-labeled telomere probe. Gels were exposed overnight on a phosphoimager cassette and analyzed on a STORM 840 Phospholager (GE Healthcare) using ImageQuant software (GE Healthcare).

Detection of telomerase activity. Telomererase activity was detected using the TRAPEze kit (Millipore, Billerica, MA), according to the manufacturer's recommendations. Briefly, 100,000 cells were collected and lysed in CHAPS buffer, and 1,000 or 500 cells were used in the telomerase extension for 30 min at 30°C followed by a two-step PCR at 94°C for 30 s and 59°C for 30 s for two cycles in a thermocycler (Eppendorf, Westbury, NY). Samples were loaded onto a 12.5% nondenaturing PAGE gel in 0.5× Tris-borate EDTA and run at 300 volts for 2 h. Gels were fixed and exposed on a phosphoimager cassette and analyzed using a STORM 840 Phospholager and ImageQuant software.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.2), 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA] for 30 min on ice and cleared by centrifugation. Protein quantitation was quantified using Bio-Rad detergent-compatible protein assay reagent (Bio-Rad Laboratories, Hercules, CA), and 100 μg of total cell extract were separated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane using standard procedures. The nitrocellulose was blocked in 5% powdered milk in TBST and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. Primary antibodies were Xrcc3 antibody (1:1,000 dilution; Novus Biologicals, Inc., Littleton, CO or Nuventa Biopharmaceuticals Corp., San Diego, CA), Nbs1 (1:1,000 dilution; Novus Biologicals), and actin (1:5,000 dilution; Abcam, Inc., Cambridge, MA). Blots were washed thrice for 10 min in TBST and probed with mouse or rabbit secondary antibodies 1:5,000 secondary antibody for 1 h followed by three 10-min washes in TBST and then developed using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Gels were stripped and reprobed with actin antibody as a loading control. Quantitation of blots was carried out using ImageQuant software and expressed as a percentage of controls. The relative expression of Xrcc3 was determined in three independent Western blots of mock-infected controls and Nbs1 in human ALT cells using stable expression of shRNAs. Western blot of Xrcc3 or Nbs1 protein expression in GM847 and WI-VA13 ALT cells stably expressing shRNAs targeting (A) Xrcc3 or (B) Nbs1. Protein expression was quantified from multiple blots using ImageQuant software and normalized using actin as the loading control. C, Xrcc3 expression after shRNA knockdown in GM847 and WI-VA13 ALT cells expressed as a percentage of mock-infected control cells. D, Nbs1 expression after shRNA knockdown in GM847 and WI-VA13 ALT cells expressed as a percentage of mock-infected control cells.
Results

Stable knockdown of Xrcc3 and Nbs1 in human ALT cells using shRNAs. We selected three well-characterized ALT cell lines to examine the role of Nbs1 and Xrcc3 in telomere maintenance and t circle formation. GM847 and WI-Va13 are laboratory-derived cells originally created from spontaneously immortalized skin and lung fibroblasts after SV40 infection. The third, Saos2, was a nonlaboratory-derived human osteosarcoma cell line.

Two shRNA target sequences were designed to inhibit Xrcc3 using Dharmacon software, whereas Nbs1 and scrambled shRNA target sequences have been reported previously (26, 27). All shRNA sequences, including a scrambled nonspecific shRNA, were cloned into the pSUPER.retro system and introduced into the three different ALT cell lines. Retroviral constructs were used to introduce shRNAs because changes in telomere length often occur gradually over many population doublings and may require long-term knockdown of protein function to expose a telomere length phenotype (28).

We monitored protein expression in each cell line using Western blot analysis. Both shRNAs targeting the Xrcc3 transcript successfully reduced expression of Xrcc3 protein in the GM847 cell line (Fig. 1A). The level of protein knockdown was similar between the two shRNA target sequences selected for these studies referred to as Xrcc3 shRNA1 and Xrcc3 shRNA2. Xrcc3 shRNAs reduced protein expression in GM847 cells by 78 ± 15% and 78 ± 18% with Xrcc3 shRNA1 and Xrcc3 shRNA2, respectively, when compared with GM847 control cells (Fig. 1A and B). These shRNAs also reduced Xrcc3 protein levels in the second ALT cell line WI-Va13 where Xrcc3 protein expression was reduced by 51 ± 21% and 70 ± 2% by shRNA1 and shRNA2, respectively (Fig. 1A and B). Throughout the study, our ALT cell lines were periodically monitored by Western blot analysis to ensure that Xrcc3 protein expression was continually inhibited by the stable expression of the shRNA sequences (data not shown).

Nbs1 was targeted in a similar manner to Xrcc3 using stable expression of previously validated Nbs1 shRNA sequences (26, 27). The two different shRNA target sequences were introduced along with appropriate controls into GM847, WI-Va13, and Saos2 cells. Nbs1 shRNA1 and shRNA2 resulted in substantial knockdown of Nbs1 protein expression by 78 ± 5% and 83 ± 5% of that observed in the GM847 controls (Fig. 1B and D). The knockdown effect in WI-Va13 cell line was more moderate but expression was still efficiently reduced by 69 ± 37% and 23 ± 16% by shRNA1 and shRNA2, respectively (Fig. 1B and D).

These cell lines were maintained in log-phase growth and their proliferation rate was determined (Fig. 2B and C). Knockdown of Xrcc3 had no observable effect on the viability or proliferation rate of the ALT cells in five of six established cell lines (Fig. 2B and C); whereas, the growth rate in cells expressing Nbs1 shRNAs was moderately slowed. The proliferation rate for GM847 control cells was 0.78 population doublings daily but dropped to 0.66 and 0.63 population doublings daily in cells expressing Nbs1 shRNA1 and shRNA2, respectively (Fig. 2B). This is an overall slowing of growth by ~20% in the GM847 cells. Similar results were observed in the majority of other cell lines containing Nbs1 shRNA (Fig. 2B; data not shown).

Telomere length in ALT cells after knockdown of Nbs1 or Xrcc3. Genomic DNA was isolated from GM847 and WI-Va13 cells using ImageQuant software and normalized using the expression of actin as a loading control. The expression levels in control cells were arbitrarily set as 100%, and the relative expression of Xrcc3 to actin in cells stably expressing Xrcc3 shRNAs were expressed as a percentage of these control cells.
~ 4 weeks after infection with shRNA retroviral constructs, and telomere lengths were assessed using telomere restriction fragment (TRF) analysis. The telomeric signal was observed as a smear on the gel, which represents the heterogeneous telomere lengths within a cell and within the population.

In general, TRF lengths were not appreciably different after expression of shRNAs directed at Xrcc3 in these two ALT cell lines, whereas appreciable changes were observed in GM847 cells expressing Nbs1 shRNAs (Fig. 2A and B). This was reflected by a shift in the telomere length distribution of Nbs1 shRNA1 and the diminished signal intensity in both the Nbs1 shRNA1 and the Nbs1 shRNA2 lanes. Further analysis of multiple pulse field gels quantified using ImageQuant software suggested that there were significant changes in total telomeric signal in GM847 cells containing Nbs1 shRNAs. This resulted in an overall reduction of telomeric signal intensity to 20 ± 3% and 26% ± 14% of the control lanes. However, this shortening was limited to the GM847 cells and was not observed in the WI-Va13 cell line in response to Nbs1 shRNAs.

**Extrachromosomal telomere repeat circles are dependent on Xrcc3 and Nbs1.** Human ALT cells contain t circles that are thought to reflect the products of t loop homologous recombination (20, 21). To establish the role of Xrcc3 and Nbs1 in t circle formation, we used two-dimensional gel electrophoresis to visualize t circles in the human ALT cells in the presence or absence of Xrcc3 or Nbs1 shRNAs. In these experiments, circular telomeric DNA was detected using two-dimensional gels as used in our recent report describing such DNA in ALT cells (21). In GM847 cells expressing a nonspecific scrambled shRNA or an empty vector, the linear telomeric DNA falls along a prominent arc, whereas the telomeric DNA circles form a slower migrating arc above the linear DNA (Fig. 3B). However, here, we observed that

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**Figure 3.** Loss of extrachromosomal t circles in GM847 and WI-Va13 cells after knockdown of Xrcc3 and Nbs1. A, model of the migration of linear and circular DNA on two-dimensional gels. B, telomere restriction fragments of ALT cells containing Nbs1, Xrcc3, or nonspecific shRNAs were separated by two-dimensional PFGE and probed with a 32P-labeled telomere-specific probe. Telomeric DNA is observed predominately as linear double-stranded DNA with a circular telomeric DNA being observed as a second arc of weaker intensity above the bulk telomere signal in mock-infected and nonspecific shRNA controls. Arrows, circular telomeric DNA.
in cells that stably express Xrcc3 shRNA1 or shRNA2, the arc representing the t circles was absent or undetectable despite a strong linear telomeric signal (Fig. 3B). Similar results were also observed in the WI-Va13 ALT cell line (Fig. 3; data not shown). These findings are consistent with a role of Xrcc3 in t circle formation in ALT cells. A similar analysis was carried out on ALT cells expressing Nbs1 shRNA. Here too, there was no detectable t circle arc in the DNA from these GM847 or in WI-Va13 cells expressing Nbs1 shRNAs.

Because GM847 and WI-Va13 are laboratory-derived cell lines, we extended our study to include the nonlaboratory-derived human osteosarcoma cell line Saos2 to determine if these proteins are also required in a cell line derived from a human cancer. The effects of Xrcc3 and Nbs1 shRNAs in Saos2 polyclonal populations were moderate; therefore, we isolated and screened multiple clonal cell lines of Saos2 cells that had significantly reduced Xrcc3 or Nbs1 expression. We established clonal cell lines after infection with Xrcc3, Nbs1, or scrambled shRNAs as described above, and protein expression was measured by Western blot analysis. Several of these clones had significantly reduced expression of Nbs1 and Xrcc3 and these were maintained in culture until a sufficient number of cells could be collected for telomere length and t circle analysis (approximately 6–8 weeks). DNA was isolated from these Saos2 clones and monitored for t circles by two-dimensional gel electrophoresis. These cells containing shRNAs directed at Nbs1 and Xrcc3 cells lacked detectable t circles, whereas those infected with scrambled shRNA or empty vector had strong t circle arcs (Fig. 4; data not shown). In addition, these clones had significantly shorter telomere lengths compared with the mock-infected controls (Fig. 4; data not shown).

ALT cells can proliferate in the absence of t circles and telomerase. Cells expressing Xrcc3 shRNAs continued to proliferate and only modest effects were observed in the presence of Nbs1 shRNAs. This suggests either that a detectable t circle arc was not a requirement for viability of human ALT cells or that cells may have activated another pathway of telomere maintenance.

To determine if the disruption of t circles in the ALT pathway results in the activation of the telomerase pathway, we used the PCR-based telomeric repeat amplification assay to test for telomerase activity in cells expressing Nbs1 and Xrcc3 shRNAs. All of our ALT cell lines were tested more than 2 months after shRNAs were introduced. We used the telomerase-positive HeLa cervical cancer cell line as a positive control and this resulted in a robust telomerase signal, observed as a ladder representing the PCR-amplified telomerase extension products. However, all of the ALT cells used in this study lacked any detectable telomerase activity even when an excess of cells were used in the assay (data not shown), suggesting that telomeres were not being maintained by telomerase expression.

Discussion

In this study, we provide evidence that Nbs1 and Xrcc3 are directly involved in the generation of t circles in human ALT cells. This was shown by the loss of t circle arcs on two-dimensional pulse field gels in cells with reduced Nbs1 or Xrcc3 expression. Furthermore, these cells continued to divide in absence of a detectable t circle arc and without activation of telomerase activity throughout the time course of this study. This was an unexpected observation and suggests that the maintenance of a pool of t circles is not a requirement for the viability of ALT cells. This finding provides important insight into the mechanism underlying t circle generation in human ALT cells and has direct implications for the treatment of ALT-positive tumors. This suggests that t circles are a poor diagnostic marker of the ALT pathway and poor target for cancer therapy because it is possible for ALT cells to survive in the absence of t circles.

The finding that ALT cells do not require t circles for telomere maintenance and survival does not imply that they are not critical
for establishment of the ALT phenotype. Indeed, the roll and spread model of telomere maintenance from studies in Kluyveromyces lactis predicts that these large t circles rapidly elongate one (or a few) telomere that could be used in telomere recombination events to spread long tracts of telomeric sequence onto neighboring telomeres (29). Therefore, synthesis of t circles may be critical during the early stages of immortalization during the establishment of the ALT pathway.

Xrc3 is one of five Rad51 paralogs that form two distinct complexes in vivo, one complex is composed of Rad51B-Rad51C-Rad51D-Xrc2 and the other Rad51C-Xrc3 (30). Two of these Rad51 paralogs, Rad51D and Xrc3, have been implicated previously in telomere biology (20, 31). However, it is unclear if any other Rad51 paralogs colocalize with telomeres or are constituents of APBs, but it is a distinct possibility given that Rad51D forms a large heteromeric complex with Rad51B-Rad51C-Xrc2 in vivo (30). Xrc3 was recently implicated in t loop homologous recombination in cells expressing TRF2Δnt, resulting in the appearance of t circles in normal cells (20). We have also shown that Xrc3 in complex with Rad51C can recognize and bind Holliday junction structures in vitro; in addition, this complex is associated with a Holliday junction resolvase activity in mammals (32). Together, these data support a model in which Rad51C and Xrc3 may be involved in recognizing and resolving Holliday junction-like structures in vivo. Because the base of the t loop closely resembles a Holliday junction, it is conceivable that this complex is also capable of recognizing and resolving t loops structures into t circles in ALT cells.

Recently, two independent studies have shown that Nbs1 is required for the formation of functional APBs and telomere maintenance in ALT cells (28, 33). In this study, we provide evidence that the disruption of Nbs1 also results in the loss of t circles, another key feature of human ALT. Interestingly, the loss of Nbs1 function and the subsequent loss of these three key features of ALT do not seem to be lethal to ALT cells, at least during the 60 to 90 population doublings observed in this and other studies (28). This is surprising given that previous reports suggest that APBs and possibly t circles participate in the ALT pathway and appear during the onset of ALT and for APBs disappear when the ALT mechanism is repressed (19, 34).

In telomerase null yeast, there is more than one type of survivor, and these subtypes are referred to as type I and type II survivors (35). Type II survivors maintain telomeres in a manner that closely resembles the telomere maintenance mechanism active in human ALT, whereas the type I survivor pathway is distinct and involves amplification of subtelomeric Y elements (36). Until recently, there was little evidence of any subtypes of the human ALT pathway and these cells were classified by a collection of common features, including the lack of detectable telomerase activity, the presence of heterogeneous telomere lengths, APBs, and the presence of abundant t circles (37). However, there is now emerging evidence that there are telomerase-negative human cells that lack some or all of these key ALT features, suggesting that there may be additional subtypes of human ALT. The telomerase null cell line AG11395 is an example of one of these ALT cell lines. It lacks APBs yet still exhibits other common characteristics of ALT cells, including dynamic telomeres and the presence of t circles (38, 39). In contrast, the ALT cell line, C3-c16, lacks both APBs and t circles (26). This latter ALT cell line adds further independent support to our data that ALT cells can exist in the absence of t circles and that this phenomenon can be seen in tumor-derived cells (24). Indeed, AG11395 cells may be the first human ALT cell line that parallels type I yeast survivors, whereas C3-c16 that lacks subtelomeric amplification may represent a novel third class of human ALT cells unlike type I or type II yeast survivors (24).

In this study, we discovered that ALT cells expressing t circles can continue to proliferate after knockdown of two recombination proteins that result in the subsequent loss of t circles, without significant effect on growth and in most cases telomere length changes. It is unclear why some ALT cells experience more dramatic telomere length changes, whereas others are less sensitive to the effects of Nbs1 or Xrc3 shRNAs. It is possible that different ALT cells undergo more subtle changes in telomere length that occur gradually with continued culture. Indeed, a previous study reported gradual telomere shortening in ALT cells in response to disruption of Nbs1 function that occurred over 120 days in culture (28). Therefore, it is possible that with continued proliferation, telomere length changes would become more apparent. Of course, it remains possible that very low levels of t circles in these cells that are undetectable by the gel analysis are still capable of maintaining telomeres in these cells. Therefore, a more significant effect may be produced by simultaneously targeting both Nbs1 and Xrc3 with shRNAs, perhaps resulting in a complete loss of the ALT phenotype and a more rapid telomere shortening phenotype. Nevertheless, all cells irrespective of their telomere length showed a loss of the pool of t circles by two-dimensional gels and continued to proliferate without the activation of telomerase.

Although telomerase has been studied extensively as a target of cancer therapies and possible diagnostic tool, there remains a lack of suitable therapeutic targets for the ALT pathway. Considering the high incidence of ALT in certain tumors, this pathway needs further exploration to determine the specific requirements of ALT to develop functional diagnostic markers and putative therapeutic targets that will be problematic in the absence of a common marker that is expressed in these cells (24, 38).

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