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# The Presence of Telomere Fusion in Sporadic Colon Cancer Independently of Disease Stage, *TP53/KRAS* Mutation Status, Mean Telomere Length, and Telomerase Activity

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## Abstract

Defects in telomere maintenance can result in telomere fusions that likely play a causative role in carcinogenesis by promoting genomic instability. However, this proposition remains to be fully understood in human colon carcinogenesis. In the present study, the temporal sequence of telomere dysfunction dynamics was delineated by analyzing telomere fusion, telomere length, telomerase activity, hotspot mutations in *KRAS* or *BRAF*, and *TP53* of tissue samples obtained from 18 colon cancer patients. Our results revealed that both the deficiency of p53 and the shortening of mean telomere length were not necessary for producing telomere fusions in colon tissue. In five cases, telomere fusion was observed even in tissue adjacent to cancerous lesions, suggesting that genomic instability is initiated in pathologically non-cancerous lesions. The extent of mean telomere attrition increased with lymph node invasiveness of tumors, implying that mean telomere shortening correlates with colon cancer progression. Telomerase activity was relatively higher in most cancer tissues containing mutation(s) in *KRAS* or *BRAF* and/or *TP53* compared to those without these hotspot mutations, suggesting that telomerase could become fully active at the late stage of colon cancer development. Interestingly, the majority of telomere fusion junctions in colon cancer appeared to be a chromatid-type containing chromosome 7q or 12q. In sum, this meticulous correlative study not only highlights the concept that telomere fusion is present in the early stages of cancer regardless of *TP53/KRAS* mutation status, mean telomere length, and telomerase activity, but also provides additional insights targeting key telomere fusion junctions which may have significant implications for colon cancer diagnoses.

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## Introduction

Telomeres are repetitive structures at the end of chromosomes that are essential for maintaining and protecting the chromosomes from degradation and end-to-end fusion [1,2]. Many studies suggest that the loss of telomere function leads to genomic instability [3–7]. Telomere dysfunction could be produced by critical telomere erosion, by disruption of various telomere-capping proteins, by stalling telomere replication, by epigenetic changes in subtelomeres, or by prolonged mitotic arrest [8–13]. The production of a broken chromosome with the dysfunctional telomere is mutagenic, because fusion of these dysfunctional telomeres propagates a breakage-fusion-bridge cycle during mitosis that results in chromosome rearrangements, deletions or amplifications. Thus, telomere dysfunction is likely a key event initiating genomic instability leading to cancer formation. Telomere integrity is mediated in part by the reverse transcriptase telomerase, which adds telomeric DNA repeats *de novo* at the ends of chromosomes ([14,15]). In human cells, telomerase activity has been shown to be very low or absent in non-malignant somatic cells. In contrast, it is activated in stem cells, germ cells, and nearly 90% of human cancer cells. Collectively, these observations imply that the immortality conferred by telomerase plays a key role in cancer development [16–18]. Telomerase is therefore required for the infinite proliferation of almost all cancer cells [19].

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Transgenic murine models have demonstrated that dysfunctional telomeres (from telomerase deficiency) along with the incomplete p53-dependent DNA damage response pathway (from p53 deficiency) lead to increased chromosome end-to-end fusions, resulting in the massive genomic instability necessary for cancer initiation [20,21]. Telomere dysfunction can also be considered as a barrier to cancer development in the presence of intact p53-cell cycle checkpoint response [22]. Therefore, these data suggest that telomere dysfunction plays a dual role in carcinogenesis. Recent murine studies also provide evidence that telomere dysfunction (induced either by telomerase deficiency or ectopic expression of dominant-negative trf2) promotes chromosomal instability that drives the early stage of carcinogenesis, and subsequent telomerase activation is critical to cancer progression including metastasis [23,24]. Although murine telomerase-knockout models have been significantly useful in identifying and characterizing the major basic biology of telomeres and telomerase, it is important to note that there are several fundamental differences between laboratory mice and humans in telomere biology [25–27]. For example, laboratory mice have about more than four-fold longer telomere length but a 40-fold shorter life-span than humans. Telomerase is expressed in almost all organs of mice, while absent or not detectable in most organs of humans. In addition, only p53 function is required for the replicative arrest of murine cells but inactivation of both the p53 and p16 pathways are needed for human cells to become malignant [28,29]. There are obviously environmental differences between human life in the world and murine life in the animal facility cage. Thus, it is critical to study carefully the complexity of human carcinogenesis by contrasting the distinctive features of telomerase and telomere biology in mouse and human carcinogenesis.

Human colorectal carcinogenesis is a well-characterized disease model for identifying genetic disposition of cancer pathogenesis [30]. Approximately 85% of colorectal cancers are characterized by a chromosome instability (CIN) phenotype, which displays an increased frequency of structural and numerical chromosomal changes. It is thought that *KRAS* (not *BRAF*) and *TP53* gene mutations are linked to the CIN-associated carcinogenesis [31–34]. A number of studies have reported that accelerated telomere shortening is observed in colorectal cancer lesions when compared to adjacent non-cancerous lesions [35–37]. While telomere shortening seems to be involved in the process of cancer progression, it remains unclear whether or when these short telomeres actually become dysfunctional. Interestingly, a recent study shows that extensive telomere erosion is associated with large-scale chromosome rearrangements in polyps obtained from familial adenomatous polyposis patients with *APC* gene defects. These data propose that the combination of short telomeres together with *APC* gene alterations in polyps may lead to chromosome instability, potentially driving clonal evolution and colorectal cancer progression [38]. Nonetheless, it is apparent that telomere length is not the sole factor determining the fusogenic behavior of human telomeres in cells, but other biological and physiological changes also cause the telomere fusion event [12,39–46]. There is a lack of conclusive data as to the degree and nature of telomere dysfunction that is linked to human colorectal cancer.

Telomere fusion (or association) has traditionally been detected by cytogenetic analysis using a significant amount of proliferating cells to obtain metaphase chromosomes, making it difficult to study kinetics and pathogenetic significance of telomere fusion events in large cohorts, especially in tissue samples. Alternatively, the anaphase bridge index is often measured in tissue sections as a surrogate marker of telomere dysfunction. It has been reported that there are 15% to 20% of anaphase bridges in tissue sections ranging from high-grade dysplasia to colon cancer, while there are less than 1% of anaphase bridges in adenoma [47]. However, a temporal relationship between telomere dysfunction dynamics and the known genomic and phenotypic characteristics of human colorectal cancer development has not yet been fully established. It is also known that the formation of anaphase bridges is associated with other events, such as cohesin defects [48] or internal double-strand DNA breaks [49]. Therefore, to overcome these technical difficulties of detecting and analyzing telomere fusions directly within human solid tumor tissue, we have developed a new molecular technique (called Telomere-Associated Repeat fusion PCR "TAR-fusion PCR" assay [50]). This assay indeed detected telomere fusions in early breast cancer such as ductal carcinoma in situ (DCIS), providing strong evidence for the occurrence of telomere dysfunction during breast cancer development [50].

**Table 1.** Patient Characteristics.

| Case No. | Age | Sex | TMN | Stage | Differentiation | Location | QC[a] (% tumor) |
|---|---|---|---|---|---|---|---|
| 1012–35 | 67 | F | TisMxN0 | 0 | Moderately | Sigmoid | 80 |
| 811–15 | 88 | M | T1MxN0 | I | Moderately | Ascending | 100 |
| 1105–08 | 50 | M | T3MxN0 | IIB | | | 60 |
| 1010–39 | 55 | F | T3MxN0 | IIB | Moderately | Ascending | 80 |
| 1005–69 | 67 | M | T3MxN0 | IIB | Poorly | Sigmoid | 70 |
| 1008–15 | 80 | F | T3MxN0 | IIB | | | 70 |
| 807–54 | 89 | F | T3M0N0 | IIB | Moderately | Sigmoid | 70 |
| 1012–31 | 89 | M | T3MxN0 | IIB | | | 50 |
| 903–22 | 78 | M | T4MxN0 | IIB | Moderately | | 70 |
| 1106–29 | 54 | M | T4bMxN0 | IIB | Moderately to poorly | | 60 |
| 1005–62 | 60 | M | T3MxN1 | IIIB | Poorly | | 50 |
| 609–08 | 65 | F | T3MxN1 | IIIB | Moderately | | 65 |
| 114–30 | 65 | M | T3MxN1b | IIIA | | Cecum | 80 |
| 908–41 | 65 | F | T4MxN1 | IIIB | Poorly | | 55 |
| 708–50 | 70 | M | T3M1N1 | IIIB | Poorly | Cecum | 60 |
| 910–44 | 47 | M | T3MxN2 | IIIC | Moderately | Sigmoid | 60 |
| 1004–70 | 68 | F | T3MxN2 | IIIC | Moderately | | 60 |
| 808–41 | 60 | M | T4MxN2 | IIIC | Well–differenced | | 70 |

Group above dashed line indicates lymph node non-invasive cases.

[a] QC, histologic quality control assessment of tissue samples.

**Table 2.** Primer Sequences.

| Name | Sequences (5′ to 3′) |
|---|---|
| Gene mutation analysis | |
| TP53_exon 5-6_forward | cacttgtgccctgactttca |
| TP53_exon 5-6_reversed | ggcccttagcctcgtaagc |
| TP53_exon 5 for seq | aaccagccctgtcgtctct |
| TP53_exon 6 for seq | agagacgacagggctggtt |
| TP53_exon 7-8_forward | ctgcttgccacaggtctcc |
| TP53_exon 7-8_ reversed | taactgcacccttggtctcc |
| TP53_exon 7 for seq | tggaagaaatcggtaagaggtg |
| TP53_exon 8 for seq | gcttcttgtcctgcttgctt |
| KRAS_c.12/13_forward | cgtctgcagtcaactggaat |
| KRAS_ c.12/13_reversed | ccctgacatactcccaagga |
| KRAS_c.12/13 for seq | gtatcaaagaatggtcctgc |
| KRAS_c.61_forward | tcatctttggagcaggaaca |
| KRAS_ c.61_reversed | cactgctctaatcccccaag |
| KRAS_c.61 for seq | ggtgcactgtaataatccag |
| BRAF_V600E_forward | ggaaagcatctcacctcatcc |
| BRAF_V600E_ reversed | gaacactgatttttgtgaatactgg |
| BRAF_V600E for seq | catttcaagccccaaaaatct |
| Telomere qPCR | |
| Ref_1_forward | ggtgatgggatttccattgatg |
| Ref_1_reversed | cttcattgacctcaactacatgg |
| Ref_2_forward | gggaagctcactggcatgg |
| Ref_2_reversed | ttcttgatgtcatcatatttggcagg |
| Telg[a] | acactaaggtttgggtttgggtttgggtttgggttagtgt |
| Telc[a] | tgttaggtatcccctatccctatccctatccctatccctaaca |
| HBG1[b] | gcttctgacacaactgtgttcactagc |
| HBG2[b] | caccaacttcatccacgttcacc |
| 36B4u[b] | cagcaagtgggaaggtgtaatcc |
| 36B4d[b] | cccattctatcatcaacgggtacaa |

[a] [16].
[b] [15].

In this present study, we investigated the relationship between known genetic alterations and the dynamic of telomere dysfunction in human colon cancer tissues and adjacent non-cancerous tissues by determining telomere fusion, telomere length, and telomerase activity. These correlative studies demonstrate that a majority of colon cancer specimens have telomere fusion regardless of the status of hotspot mutations in both *TP53* and *KRAS* genes. Mean telomere shortening and weak telomerase activity were not associated strongly with the presence of telomere fusion, implying that telomere fusion is a key genetic aberration resulting from telomere dysfunction. Moreover, the results from the telomere fusion junction analyses suggest that the nature of telomere fusion junctions may be linked to neoplasticity.

## Materials and Methods

### *Tissue Samples*

Tissues specimens were obtained at the time of surgery, immediately frozen in liquid nitrogen, and stored in liquid nitrogen until use. All patients had only surgery without combination therapies. Paired frozen tissues (100–150 mg) from sporadic colon cancer tissues (n = 18) and their adjacent tissues (n = 18) were obtained from the tissue bank at the Indiana University Simon Cancer Center. All cases were reviewed by pathologists to assess tumor histology. Each pathologically normal adjacent tissue was harvested at least 2 cm distal from the cancer tissue. The purity of each specimen was shown as % tumor in Table 1. The study was approved by the institutional review board of the Indiana University.

### *DNA Isolation*

Genomic DNA from each frozen tissue was isolated by a salt precipitation method as previously described [51]. Briefly, about 20 mg of each frozen tissue fragment was minced quickly in cold PBS (–) and the PBS (–) was removed by centrifuging at 3500 × *g*. The pellets were resolved in lysis buffer (20 mM Tri-HCl/pH 8.2, 10 mM EDTA, 400 mM NaCl, 0.5% SDS, 0.05 µg/µl proteinase K) and incubated overnight at 56°C. The DNA solution was centrifuged for 15 min at 9600 × *g* after adding the one-fourth volume of saturated NaCl. The supernatant was transferred to a new tube and the DNA

**Table 3.** Summary of Mutations and Telomere Dysfunction Dynamics.

| Case No. | TP53 Exon 5–8 | KRAS Codons 12&13[a] | BRAF V600E | Telomere fusion junction[b] | | Relative telomere erosion[c] | TRAP activity (%)[d] | |
|---|---|---|---|---|---|---|---|---|
| | | | | Tumor | Adjacent | | Tumor | Adjacent |
| 1012–35 | | | | 7q–7q | | 0.874 | 49.92 | 8.69 |
| 811–15 | | GtTGGC | | other | | 0.559 | 10.53 | 4.04 |
| 1105–08 | Codon 245 (GGC>AGC) | GtTGGC | | | | 0.786 | 24.18 | 13.69 |
| 1010–39 | Codon 132 (AAG>AGG) | aGTGGC | | other | | 0.730 | 87.30 | 1.94 |
| 1005–69 | | | | 7q–7q | | 1.060 | 13.84 | 2.12 |
| 1008–15 | | | + | | | 0.7053 | 104.63 | 14.71 |
| 807–54 | Codon 199 (GGA>AGA) | | + | 7q–7q | | 0.753 | 10.68 | 4.49 |
| 1012–31 | Codon 162 (ATC>AAC) | | | 7q–7q, 4pq–XpYp | | 0.731 | 7.79 | 1.31 |
| 903–22 | | | + | other | other | 0.501 | 318.58 | 1.38 |
| 1106–29 | | | | 4pq–XpYp | | 0.721 | 8.13 | 3.64 |
| 1005–62 | | | | 7q–7q, other | 7q–7q, other | 0.400 | 15.92 | 8.85 |
| 609–08 | | | + | 7q–7q | | 0.706 | 27.13 | 3.29 |
| 1104–30 | | GtTGGC | | other | other | 0.796 | 20.48 | 3.43 |
| 908–41 | Codon 131 (AAC>GAC) | | + | 4pq–XpYp | 7q–7q | 0.261 | 64.11 | 11.76 |
| 708–50 | | | + | | | 0.701 | 70.53 | 1.80 |
| 910–44 | Codon 282 (CCG>CTG) | | | 7q–7q, 4pq–XpYp | | 0.567 | 43.14 | 9.72 |
| 1004–70 | Codon 135 (TGC>TGG) | GGTGaC | | 7q–7q | | 0.361 | 85.23 | 5.55 |
| 808–41 | | | | 7q–7q | 7q–7q | 0.417 | 0.76 | 0.70 |

Group above dashed line indicates lymph node non-invasive cases.
[a] Mutation shown in lower case. There were no mutations found in codon 61.
[b] 7q-7q and 4pq-XpYp indicate a fusion junction containing DNA sequences shown in Figure 4, *A* and *B*, respectively. Other means a fusion junction containing chromosome XpYp and one from other chromosomes. Identical fusion junctions between paired cancer and adjacent DNA samples were shown in *italic*.
[c] Graph is shown in Figure 2*C*.
[d] Graph is shown in Figure 3*C*.

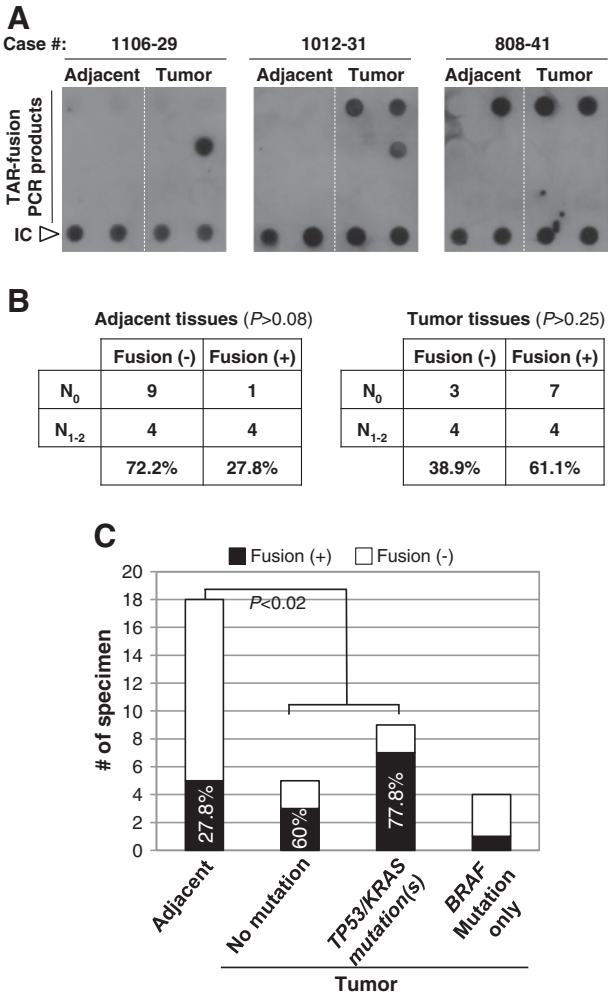


**Figure 1.** Telomere fusions arise prior to *KRAS/TP53* mutations and are retained during tumor progression. (A) Dot blots show representative telomere fusion assays. Each case contains six PCR reactions using TAR-fusion PCR primers and two PCR reactions using control primers. Telomere fusion junctions were PCR-amplified, denatured and dot-blotted onto membranes and hybridized to a DIG-labeled telomere probe. (B) Relationship between telomere fusion status and lymph node invasiveness. $N_0$, lymph node non-invasive case. $N_{1-2}$, lymph node invasive case. The data from tumor tissue samples represent only "neogenetic" fusion. (C) Relationship between telomere fusion status and mutation status in *KRAS/BRAF* and *TP53* genes. Student's *t* test was performed in (B) and (C).

was precipitated by adding the equal volume of isopropanol. After rinse with 70% ethanol, DNA was resolved in TE. The DNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

### *Telomere Quantitative PCR (qPCR) Assay*

Telomere length was determined using real-time qPCR [52,53] with minor modifications. All real-time PCR reactions were carried out using the LightCycler 480 (Roche Diagnostics) and Roche-brand SYBR green I master mix. Commercialized diploid DNA was used as a control template (Promega). Telomere and reference sequences were amplified using the following conditions: 95°C for 10 minutes to activate DNA polymerase, and 2 cycles each at 95°C for 10 seconds, 49°C for 10 seconds, and then 35 cycles each at 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 10 seconds. LightCycler 480 software version 1.5 was used for analysis. Primer sequence information is shown in Table 2. The reference primers minimized the effect of chromosome and/or gene copy number variation in each sample by amplifying 13 loci on eleven chromosomes including chromosome 1, 4, 5, 6, 7, 8, 12, 13, 19, 21, and X.

### *Telomeric Repeat Amplification Protocol (TRAP) Assay*

Telomerase activity was analyzed by TRAP assay as previously described [54]. Briefly, 1 μg of protein extracts was used for the assay in 50 μl reactions with Taq DNA polymerase. The PCR products were electrophoresed in a 12.5% non-denaturing polyacrylamide gel and stained with SYBR Gold dye (Life Technologies). The gels were scanned using the STORM 860 molecular imager and quantified using ImageQuant TL software (GE Healthcare Life Sciences). Signals from all telomerase ladders were summed for a net TRAP activity per sample. The data were normalized to the signal from the internal standard (IC), and then % was calculated as relative to the HeLa TRAP standard (Figure 2B; [55]). TRAP activity of 1.0 μg HeLa extract is defined as 100%.

### *Telomere Associated Repeat (TAR)–Fusion PCR Assay*

Telomere fusions were determined using TAR-fusion PCR assay [50] with minor modifications. Two-step touchdown PCR was performed in a 20 μl reaction mixture using 50 ng of DNA, multiple primers, 10% 7-Deaza-dGTP (Roche Diagnostics), and Advantage GC Genomic LA Polymerase Mix (Clontech Laboratories). Multiple PCR primers were designed within TAR1 (Telomere Associated Repeat 1)–like sequences common to many chromosomes distal regions [56]. The primer sequence information and PCR condition are shown in Tanaka et al. [50]. As an internal positive PCR control, the following primers were used for amplifying ancestral interstitial telomere head-to-head fusion within chromosome 2q13-q14.1 [57]: 5′-GCA AGG CGA GGG GCT GCA TTG CAG GGT GAG-3′, 5′-CAG CAG GGG GCG CTG GAC AGC ACT GTA AG-3′. TAR fusion PCR products were dot-blotted onto Hybond$^+$ membranes and hybridized using a DIG-labeled telomere probe (Roche Diagnostics). Six PCR reactions per each case were used for every PCR. Telomere fusion was defined as 'negative' when TAR-fusion PCR did not amplify any products using total twenty PCR reactions which correspond to more than $1 \times 10^5$ cells.

### *Analysis of Telomere Fusion Junctions*

TAR-fusion PCR products were purified by the GENECLEAN Turbo Kit (MP Biomedicals). Cloning and transformation were carried out with a TOPO TA PCR Cloning Kit (Life Technologies). TOP10 (Life Technologies) or SURE (Agilent Technologies) competent cells were used for transformation with blue/white selection. White colonies were picked and each inserted DNA was confirmed by colony PCR. More than 1 kb of inserted DNA length was selected for DNA sequencing. The sequencing service was provided by GENEWIZ after DNA isolation using Qiaprep Miniprep kit (Qiagen). Sequence alignments were performed using the University of California Santa Clara Genome Bioinformatics database (http://genome.ucsc.edu/).

### *Gene Mutation Analysis*

*TP53* mutation between exons 5 and 8, *KRAS* mutation at codons 12, 13 and 61, as well as *BRAF* mutation (V600E) at exon 15 were determined by direct sequencing. The sequencing service was provided by GENEWIZ after PCR. The primer sequence

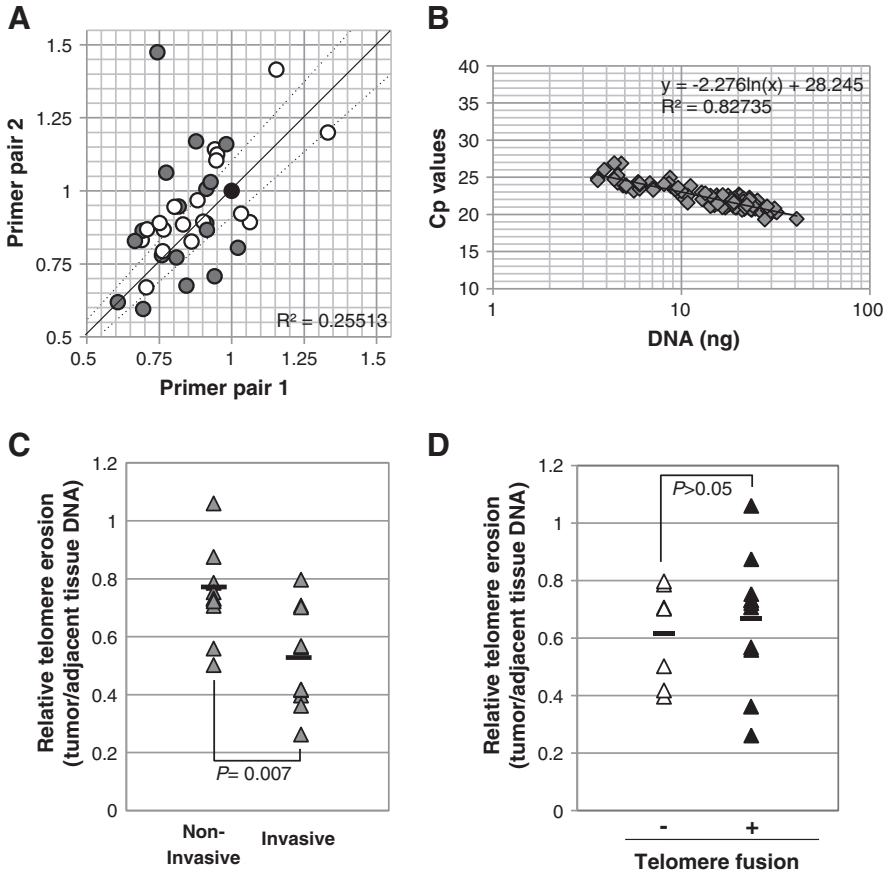


**Figure 2.** Colon cancer develops with increasing telomere shortening. (A) Influence of DNA ploidy in the existing PCR method developed by Cawthon [52,53]. Graph shows a poor correlation between two different reference primers, 36B4 and β-globin [52]. X-axis represents 36B4 primers, which encodes acidic ribosomal phosphoprotein PO and y-axis represents β-globin primers. Human diploid DNA was used as reference template (36B4:β-globin = 1:1 ratio) and relative value in each case was calculated based on the value from diploid DNA. Each circle shows human diploid DNA in black, tumor tissue DNA in gray, and adjacent tissue DNA in white, respectively. Dash line indicates ± 10% far from the 1:1 ratio. (B) Semi-logarithmic graph shows a linear correlation between input DNA from all specimens and Cp (crossing-point) value of new reference primers for improved telomere qPCR assay. The Cp is the cycle number at which the fluorescence rises appreciably above the background fluorescence. Eighteen pairs of matched tumor and adjacent tissue DNA were amplified, along with diploid DNA. (C) Change in telomere length was measured in tumor DNA compared to the matched adjacent DNA. Change in <1 represents telomere shortening in tumor DNA. Graph shows significant regression of telomere length in lymph node invasive-positive group (N = 8, *average* = 0.526) compared to lymph node invasive-negative group (N = 10, *average* = 0.742). Black bar represents *average* in each group. (D) Relationship between telomere fusion status and change in telomere length. Black bar represents *average* in each group. Paired Student's *t* test was performed.

information is shown in Table 1. A summary of the sequence analysis was shown in Table 3.

### *Statistical Analysis*

All statistical analyses were performed using Pearson correlation, Fisher's exact test, and the two-tailed Student *t* test. A *P* value of less than .05 was considered statistically significant.

## Results and Discussion

### *Relationship Between Telomere Fusion Status and Known Genetic Changes in Colon Carcinogenesis*

Determining the presence of telomere fusion is the firmest evidence that telomeres are actually dysfunctional in cells. Molecular analyses of telomere fusion in human epithelial tissue have begun since two groups have independently developed PCR-based methods to amplified telomere fusion junctions ([50,58]). Although Roger et al. reported that telomere fusion events were detected in 6 of 45 adenomatous colorectal polyps (13.3%) and 5 of 8 colorectal adenocarcinomas (62.5%), there was little discussion about the temporal sequence of telomere dysfunctions along with any other factors which may be related to the telomere fusion events [38]. In this study, we further investigated both the degree and nature of telomere dysfunctions in 18 paired tissue samples of tumor and adjacent tissue lesions from sporadic colon cancer patients. Informative characteristics of all specimens were shown in Table 1. Eight cases belonged to a lymph node invasive cancer group and the remaining 10 cases were a non-invasive cancer group. Because cancer cells are almost always intermixed with an unknown fraction of normal cells, we selected specimens with more than 50% of tumor cells and repeated experiments with multiple reactions. The TAR-fusion PCR assay is composed of two steps, multiplex PCR and dot-blot using a telomere probe. We improved the current assay by using internal control primers to rule out the possibility of false negative amplification (Figure 1*A*).

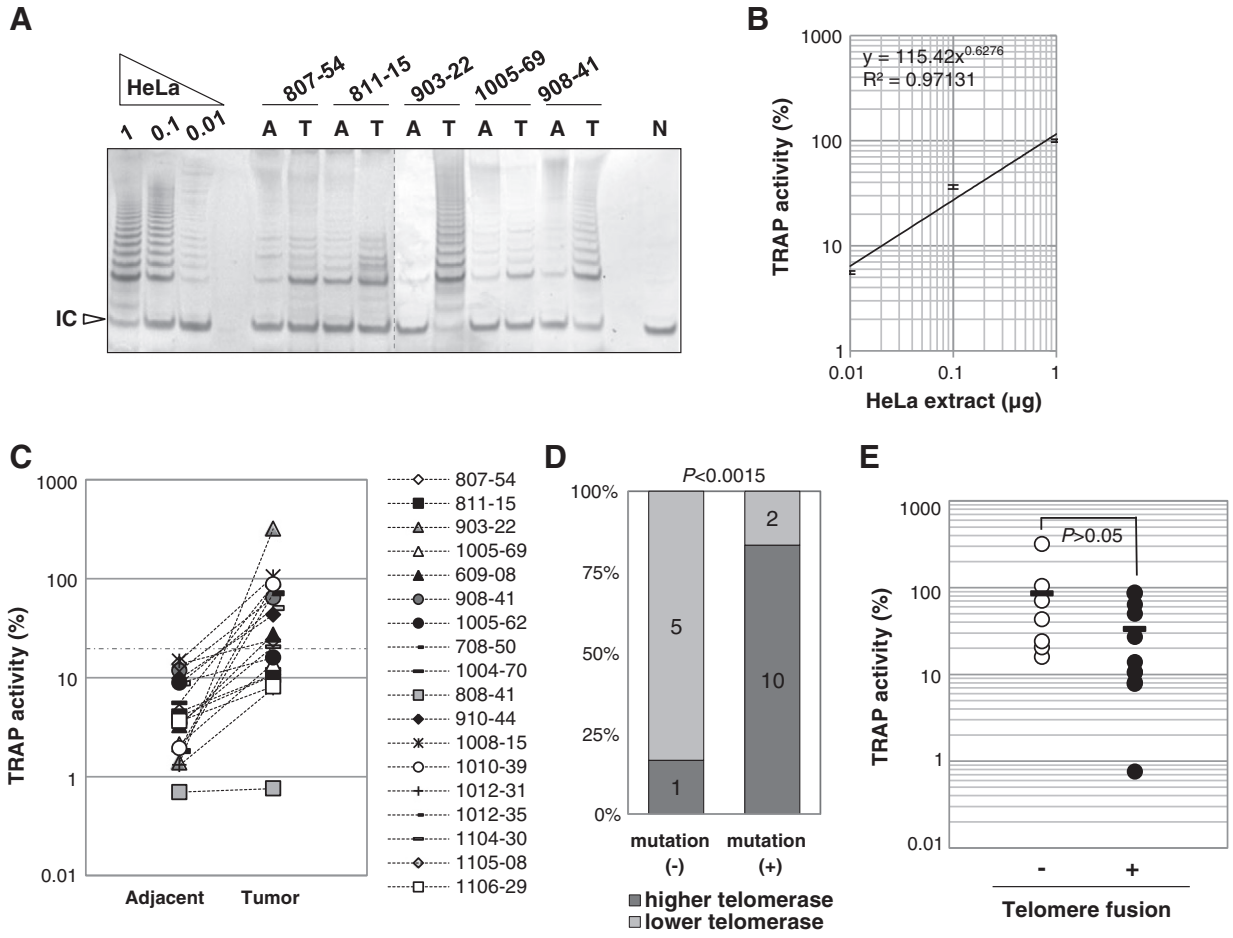


**Figure 3.** Telomerase activity is up-regulated in tumor tissue compared to the matched adjacent tissue and the up-regulation is associated with the presence of gene mutations. (A) Representative TRAP result was shown. HeLa cell extract (1 $\mu$g, 0.1 $\mu$g, and 0.01 $\mu$g) was used as a positive control. N represents no template (lysis buffer) as a negative control. IC represents internal control for PCR. A and T represent adjacent and tumor tissue, respectively. Two individual gels were combined in this image. (B) Double-logarithmic graph shows standard curve for calculating percent of HeLa's TRAP activity. The progression line closely fits the data ($R^2 = 0.971$). Data represent mean ± SEM (N = 6). (C) Semi-logarithmic graph shows that tumor tissues have higher TRAP activity than their adjacent tissues TRAP in all 18 cases. More than 20% (corresponding to 0.05–0.06 $\mu$g of HeLa extract) of TRAP activity was defined as higher. Dashed line represents 20% TRAP activity. (D) Histogram shows higher telomerase activity is significantly associated with the presence of mutations in *TP53* and/or either *KRAS* or *BRAF* genes. (E) Relationship between telomere fusion status and TRAP activity. Black bar represents *average* in each group. Paired Student's *t* test was performed.

Unexpectedly, but not surprisingly, telomere fusions were found not only in cancer tissues but also in five adjacent non-cancerous tissues (e.g., case #808-41; Figure 1, *A* and *B*, and Table 3). This result reinforced previous evidence that pathologically normal did not always mean genetically normal [59–63]. Therefore, we carefully estimated the presence of 'neogenetic' telomere fusion in each cancer group by excluding identical fusions between adjacent non-cancerous tissue and the matched cancer specimen based on the fusion junction DNA sequences (see below). While we do not understand at this point how cancer tissue contains the same fusion type as the adjacent tissue, it could be due to the fact that identical fusions resulted simply from intermixed tissues or cancer tissue evolved from a cell in the adjacent tissue.

Overall, telomere fusions were highly detectable in 61.1% of total cases independently of the lymph node invasion status (Figure 1*B*). Telomere fusions are presented in 60% of the cases without any hotspot mutations in *TP53*, *KRAS*, and *BRAF* genes, as well as 77.8% of the cases with *TP53* and/or *KRAS* mutation(s) (Figure 1*C*). These results suggest that telomere fusions arise prior to the occurrence of oncogenic/tumor suppressor mutations and are retained during a mutagenic period. In contrast, BRAF V600E mutation alone was poorly associated with the presence of telomere fusion in colon cancer (Figure 1*C*). This observation is consistent with the previous findings that BRAF V600E mutation is a reasonable indicator of CIN-negative colorectal cancer [34]. Therefore, it is speculated that cancer with a BRAF V600E mutation alone may not be involved in the telomere dysfunction-driven genomic instability pathway.

### *Change in Telomere Length Between Lymph Node Invasive and non-Invasive Colon Cancer*

The current telomere qPCR method facilitates measurement of relative telomere length in large cohorts, especially in the scope of estimating cancer risk, mortality, or natural survival rate by using peripheral leukocyte telomere length [52,53]. This method is designed to determine average telomere length normalized by a single copy gene such as human β-globin gene. Hence, this method is optimized for diploid DNA samples as a template. As expected, DNA from tumor

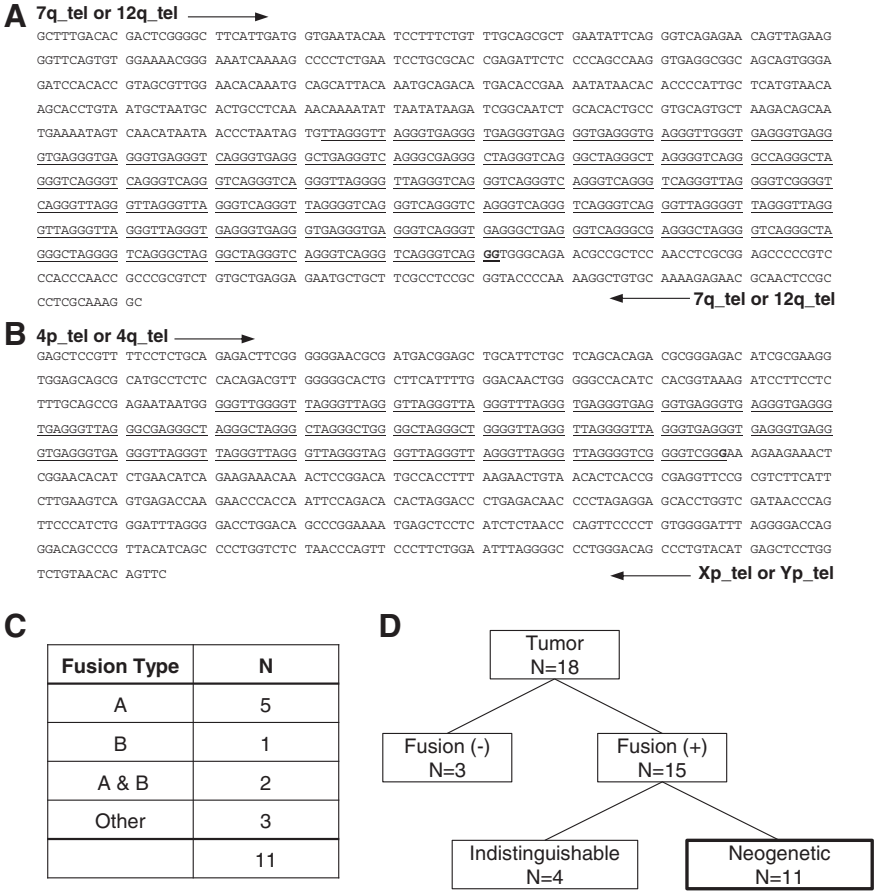


**Figure 4.** The nature of telomere fusion junctions may be linked to neoplasticity. (A and B) Representative DNA sequences of telomere fusion junctions in tumor DNA. (A) shows the sequences between 7q or 12q and 7q or 12q. (B) shows the sequences between 4p or 4q and Xp or Yp. Telomeric repeats were underscored. Microhomology at each fusion junction was indicated by *boldface*. (C) Each tumor was categorized by telomere fusion type. A and B correspond to the telomere fusion junction shown in (A) and (B), respectively. (D) Frequency of telomere fusion in tumor tissues.

tissues was not able to show one-to-one (1:1) correlation of PCR amplification between two different single copy genes (Figure 2*A*; β-globin and 36B4). This finding coincides evidence that cancer-driven DNA is no longer diploid [64–66]. Interestingly, there is no 1:1 correlation even in DNA from some of surrounding non-cancerous tissue samples, supporting previous evidence that those tissues contain aneuploid DNA [67,68]. Indeed, several studies have demonstrated that surrounding tissues no longer act normal in terms of gene expression and genetic mutation [59–63]. Therefore, to avoid the negative impact on changes in chromosome and/or gene copy number in each sample, we optimized the telomere qPCR by using new designed reference primers (Table 2). A significant correlation as 0.827 of coefficient of determination ($R^2$) was shown in a relationship between reference Cp value and template DNA amount in each sample (Figure 2*B*). The 18 paired tumor and adjacent tissue specimens were used for the telomere length measurement. The alteration of average telomere length in tumor tissues was evaluated by comparison of their adjacent non-cancerous tissues. The results revealed that the change in telomere length was affected by a degree of regional lymph node involvement ($P = .007$, Figure 2*C*). Consistent with other cancer studies [69–71], our findings suggested that colon cancer could develop with continuing telomere shortening. However, there have been observations indicating that telomere length is not correlated with tumor stage or grade in colorectal cancer [35,37,72]. These contradictions could result from interpretation without sufficient information about the purity of specimens, family history of cancer, or additional therapies besides surgery, because we now know that these factors have an impact on telomere length alteration and need to have been fully considered [73–77]. Also, some of the analyses were performed by comparing average telomere length in each group, instead of comparing age-adjusted telomere length or a ratio of telomere length in cancer tissue to corresponding non-cancer tissue in each patient. This is critical to adjust the age-dependent variation of telomere length values [78,79]. Establishing a standardized analysis technique may help resolve the different interpretations of telomere length measurement. Furthermore, there was no significant association between telomere length and telomere fusion in this study ($P > .05$, Figure 2*D*). It is notable that telomere fusion was detected even in carcinoma *in situ* (e.g., case #1012-35, Table 3). This observation is not ruled out of a causal connection between telomere shortening and telomere fusion, however instead, indicates that the sensitivity of TAR-fusion PCR as a telomere dysfunction marker could be higher than both telomere qPCR and TRAP assay (Figures 2*D* and 3*D*, also see below).

### *Timing of the Onset of Telomerase up-Regulation in Colon Cancer*

We next quantified telomerase activity carefully in each sample using TRAP assay (Figure 3*A*). After subtracting a PCR internal control (IC) signal from the intensity of net telomerase ladders,

telomerase activity was shown as a percentage (%) after normalization to the telomerase activity in HeLa cells. We confirmed that there was a linear correlation between TRAP activity and the amount of HeLa protein in a log-log plot ($R^2$ = 0.971, Figure 3*B*). In all 18 cases, we observed increasing telomerase activity independent of the degree of regional lymph node involvement (Figure 3*C*). Interestingly, when samples were divided into two groups with or without hotspot mutation(s) in *TP53* and/or either *KRAS* or *BRAF* genes, 83.3% of the mutation-positive group has higher (<20%) telomerase activity (Figure 3*D*). In contrast, only 16.7% of the mutation-negative group has higher telomerase activity. This result suggested that the up-regulation of telomerase activity was associated with the presence of known genetic changes, and corroborated an existing idea that the full activation of telomerase could be a late event in colorectal carcinogenesis [80–83]. No link between telomerase activity and telomere length was observed in this study (data not shown). An association between telomere fusion rate and telomerase activity was not statistically significant (Figure 3*E*).

### *The Nature of Telomere Fusion Junctions*

To identify the key internal structures of telomere fusion junctions, we performed the sequence analysis. The results showed that all fusion junctions lost telomeric (TTAGGG)n (or telomere-like) sequences on one end and fused to the most proximal subtelomere region of another chromosome. We identified two major fusion junction sequences in sporadic colon cancer specimens (Figure 4, *A* and *B*). Out of 11 telomere fusion-positive cancers, seven cancers had 7q to 7q (or 12q to 12q, or 7q to 12q) fusion junction (Figure 4*C* and Table 3). Interestingly, these two junctions are different from those that were found previously in chronic lymphocytic leukemia and breast cancer [50,70], proposing that the chromosomes involved in end-to-end fusions may not be randomly distributed. In other words, the telomere fusion may occur preferentially between cancer types. It is expected that identifying the target of specific chromosomes or elements responsible toward malignancy could potentially provide useful clues into targeting treatment strategies, as well as into screening cancer risk. Our result does not necessarily imply that only chromosome 7 and 12 are vulnerable to fusion in sporadic colon cancer, in part because our PCR primers do not cover all possible fusion types. Total 15 out of 18 cases contained telomere fusion, however, telomere fusion was observed even in tissue adjacent to cancerous lesions in five cases. Four of them were indistinguishable from telomere fusion found in their cancer tissues (Figure 4*D* and Table 3).

## Conclusions

This careful correlative study using paired tumor and adjacent tissue specimens corroborated the concept that telomere fusion is indeed present in the early stages of sporadic colon cancer, prior to *TP53* and/or *KRAS* mutations, critical shortening of mean telomere length, and telomerase activation in human colon cancer. In addition, the type of telomere fusion may vary with the type of cancer. Our results pave the way for further investigations to help elucidate the relationship between telomere dysfunction dynamics and human carcinogenesis. Moreover, new insights gained from each normal adjacent tissue will facilitate our further understanding that how the neighboring cells impinge on non-autonomous tumor progression.

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