Correlation of telomere length shortening with *TP53* somatic mutations, polymorphisms and allelic loss in breast tumors and esophageal cancer

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Abstract. Genomic instability caused by telomere erosion is an important mechanism of tumorigenesis. p53 plays a key role in cellular senescence and/or apoptosis associated with telomere erosion which positions p53 as a guard against tumorigenesis. The present study was undertaken to investigate the potential interactions between p53 functional mutations, polymorphisms, allelic loss and telomere erosion in 126 breast tumor patients and 68 esophageal cancer patients. Telomere length (TL) was measured by real-time quantitative PCR. Somatic mutations, polymorphisms and allelic loss in the *TP53* gene were detected by direct sequencing of both tumor and normal tissue samples. Our results showed that telomeres were significantly shorter in tumors with somatic p53 mutations compared with tumors with wild-type p53 in both breast tumors (P=0.007) and esophageal cancer (P=0.001). Telomeres of patients with minor genotype CC of rs12951053 and GG of rs1042522 were significantly shorter compared to patients with other genotypes of this single nucleotide polymorphism in esophageal cancer tissue. Furthermore, *TP53* allelic loss was detected and significantly associated with somatic mutations in both types of tumor tissues. These findings suggest that somatic p53 mutations, rs12951053 genotype CC and rs1042522 genotype GG contribute to erosion of telomeres, and *TP53* allelic loss may be one of the representations of chromosomal instability caused by telomere erosion combined with somatic p53 mutations. These results support that the *TP53* gene has a strong interaction with TL erosion in tumorigenesis.

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Key words: telomere erosion, *TP53* variations, allelic loss, breast tumor, esophageal cancer

Introduction

Telomeres are special structures consisting of a stretch of very simple tandemly repeated sequences and telomere structural proteins at the terminal of chromosomes (1). Their main function is to cap the chromosome ends and prevent chromosomal instability, while the erosion of telomeres can lead to genetic instability, a pivotal mechanism in the neoplastic process (2,3). Because of incomplete replication of the termini of linear DNA molecules, telomeric DNA is progressively lost with each cell division (4,5). Telomere shortening reaching a critically short length can activate DNA damage checkpoints and result in induction of cellular senescence (6). The first checkpoint in response to telomere shortening is a p53-dependent, permanent cell cycle arrest. p53 plays a key role in cellular senescence

Table I. Primers for PCR and sequencing.

Primer name	Primer sequence 5'-3'	Type	Amplified/sequencing fragment[a] (locus)
TP53F	ACGACGAGTTTATCAGGA	Amplifying	g.11066-g.14379
TP53R	GACCTATGGAAACTGTGAG	Amplifying	
TP53S1	ACGGCATTTTGAGTGTTAG	Sequencing	g.13294-g.14084 (exons 7-9)
TP53S2	GGATGGGTAGTAGTATGGAAG	Sequencing	
TP53R1	CCTGATTTCCTTACTGCCTCTT	Sequencing	
TP53R2	TGCTTGCCACAGGTCTCC	Sequencing	
TP53S3	TCAAATAAGCAGCAGGAGA	Sequencing	g.12338-g.12805 (exons 5-6)
TP53R3	TGCCGTCTTCCAGTTGCT	Sequencing	
TP53S4	GTGAAGAGGAATCCCAAAG	Sequencing	g.11114-g.11656 (exons 3-4)
TP53R4	CCTATGGAAACTGTGAGTGGA	Sequencing	

[a]Nomenclature according to the HGVS standards with the GenBank NC_000017.9 genomic sequence as reference.

and/or apoptosis associated with telomere dysfunction (7). It may prevent entry into mitosis with uncapped telomeres (8), and intact p53 signaling could be a prerequisite for induction of senescence and/or apoptosis in response to critical telomere shortening (9). When p53 is mutated or deleted, p53-dependent responses to telomere dysfunction are mitigated and chromosomal fusions are tolerated. This results in chromosome breakage and genomic copy number alterations (CNAs) and drives development of carcinomas (3,10). These all position p53 as the guard against tumorigenesis caused by telomere dysfunction.

TP53 is a tumor-suppressor gene, whose mutations and loss of heterozygosity (LOH) are hallmarks of most human cancers (11,12). Mutations in the coding sequence can cause dramatic defects in p53 function, and some polymorphisms in the *TP53* locus might have phenotypic manifestations (13,14). LOH has emerged as the second hit in tumor initiation which serves to inactivate or eliminate the wild-type allele at the tumor-suppressor gene locus (12,15,16). These mutations, polymorphisms or allelic loss (or LOH) that may change p53 function have a relationship with telomere erosion and tumorigenesis.

Both breast and esophageal cancers are the most common tumors. However, no study has previously investigated the relationship between *TP53* gene variants and telomere length (TL) in breast tumor and esophageal cancer. The relationships between *TP53* mutations, polymorphisms, allelic loss and TL are still largely undefined. The present study, which investigated the *TP53* gene and TL from 126 Chinese breast tumor patients and 68 Chinese esophageal cancer patients, was aimed at investigating the potential interaction between p53 functional mutations, polymorphisms, allelic loss and telomere erosion. This study may help us better understand the molecular mechanism of tumorigenesis, which should lead to improved screening and treatment of cancer.

Materials and methods

Study population. A total of 126 breast tumor patients and 68 esophageal cancer patients of Chinese ancestry were included in the present study. All of the breast tumor samples, including 45 malign and 81 benign breast tumor samples, were consecutively collected from the Yunnan Province. Breast tumor tissue and a blood sample from each patient were collected for genomic DNA extraction and genotyping. Sixty-eight esophageal cancer specimens were consecutively collected from the Henan Province. For esophageal cancer patients, each cancer tissue and normal tissue were collected for study. Written consent was obtained from all participants, in accordance with protocols approved by the institutional review board at each contributing center.

Telomere measurement. Genomic DNA was isolated from whole blood samples and tissues by standard phenol/chloroform method. Relative telomere length was measured on extracted DNA using real-time quantitative PCR (17,18) with minor modifications. Standard curves for TL and single-copy gene (reference gene) were used to transform cycle threshold into nanograms of DNA. Triplicate PCR reactions were performed in 20 μl reactions comprising 8 μl template DNA, 2 μl primer mixture and 10 μl SYBR® Premix Ex Taq™ (Takara, Dalian, China). The final telomere and 36B4 primer concentrations were 0.2 and 0.3 μM. The primer sequences were as previously described (18). The reaction mixture was initially denatured at 95˚C for 2 min followed by 40 cycles of 95˚C for 5 sec, 58˚C for 10 sec, and 72˚C for 40 sec for the 36B4 reaction, or 25 cycles of 95˚C for 5 sec, 56˚C for 10 sec, and 72˚C for 60 sec for the telomere reaction. All PCRs were performed using a 96-well formatted LightCycler® 480 Real-Time PCR system (Roche Applied Science), and results were obtained and analyzed using the LightCycler® 480 onboard software (version 1.5).

Mutational screening and genotyping of TP53. According to the *TP53* somatic mutations database (IARC *TP53* database, http://www-p53.iarc.fr, R15 release) (11), 96% of somatic mutations are located in exons 3-9 of the gene. Thus, we sequenced parts of the *TP53* gene to discover somatic mutations and other variations. Exons 3-9 and respective intron-exon boundaries were included. Primers for PCR and sequencing are listed in Table I. PCR was carried out in 25 μl of reaction containing

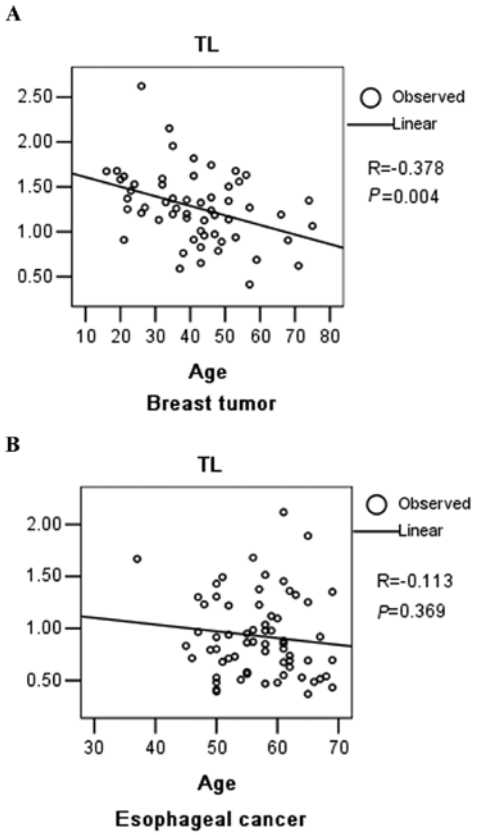

Figure 1. Correlation between telomere length and age (years) as assessed by the linear regression model. (A) Breast tumor tissue; (B) esophageal cancer tissue.

1X LA PCR Buffer II (Mg^{2+} Plus), 20 ng DNA, 0.5 μM each of the primers, 0.4 mM each of deoxynucleotide triphosphate, and 1.25 U of LA Taq DNA polymerase (Takara). The reaction mixture was denatured at 95˚C for 5 min followed by 10 cycles of 1 min of denaturation at 94˚C, 1 min of reannealing at 60-50˚C (decreased by 1˚C every cycle), and 4 min of extension at 72˚C; 25 cycles of 1 min of denaturation at 94˚C, 1 min of reannealing at 50˚C and 4 min of extension at 72˚C. The PCR was completed by a final extension at 72˚C for 10 min. The products were purified with gel extraction kits (Watson BioMedical Inc., Shanghai, China) and were subjected to direct DNA sequencing using the BigDye® Terminator v3.1 Cycle Sequencing kit and ABI PRISM 3730 sequencer (Applied Biosystems Inc., USA). Sequences were aligned and analyzed with DNAStar software package (DNAStar Inc., Madison, WI, USA). For the malign breast tumors and esophageal cancer patients, both tissues for each patient were sequenced. For the benign breast tumor patients that had variations not included in the known polymorphisms, whole blood samples were also sequenced. All somatic mutations found by direct sequencing of PCR products were confirmed by sequencing of a second, independent PCR product. All sequences were submitted to GenBank (accession no. JQ751320-JQ752243).

All polymorphisms in each individual were detected and confirmed by sequencing the corresponding regions in both tissues. Allelic loss was determined by comparing tumor and normal single nucleotide polymorphism (SNP) allele types. Linkage disequilibrium (LD) coefficient (D' and r2) and Hardy-Weinberg equilibrium P-value were estimated by Haploview software version 4.2 package (http://www.broad.mit.edu/mpg/haploview/). For each polymorphism, Hardy-Weinberg equilibrium (HWE) was tested by comparing the observed to expected genotype frequencies. All SNPs were consistent with HWE (P>0.05). Reconstruction of the *TP53* haplotypes incorporating the 7 SNPs was accomplished using Phase software. Four distinct haplotypes were observed in the study population with a frequency >1%. Each individual was assigned the best pair of haplotypes estimated by Phase software.

Statistical analysis. TL was analyzed as a continuous variable. The Mann-Whitney U test or the Kruskal-Wallis H test was used as appropriate to determine the differences in TL between different groups. Correlation curves between age and TL were estimated by linear regression model. A Chi-squared test or Fisher's exact test was used as appropriate to assess differences in allelic loss frequency between different groups and genotype distribution of each tagSNP between malign and benign breast tumor patients. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was declared at α=0.05.

Results

Breast tumors

TL and its association with somatic p53 mutations. In 124 breast tumor samples, TL was determined by real-time PCR. The mean level of TL in breast tumor tissues was 1.346 [standard error (SE) =0.039]. Mean TL of malign patients was shorter than that of benign patients, although no significant difference was observed (P=0.102). The mean TLs were 1.263 (SE=0.067) and 1.391 (SE=0.047) in malign breast tumors and benign breast tumors, respectively. The TLs of the breast tumor tissues were plotted against patient age at sampling (Fig. 1A). The negative slope of the best-fit line for breast tumor tissue indicated a decrease in the TL with age in the breast tumor patients (R=-0.378, P=0.004).

Table II shows the pattern and codon distribution of *TP53* somatic mutations in our patients. In the breast tumor patients, we found a total of 11 somatic mutations in 10 patients and 1 patient had double mutations. Therefore, the frequency of *TP53* gene somatic mutations in breast cancer was 22.7% (10/44) in our study. The proportions of different mutation types were 1/11 (9.1%) for A:T→G:C, 2/11 (18.2%) for G:C→A:T, 3/11 (27.3%) for G:C→T:A, 1/11 (9.1%) for ins and 4/11 (36.4%) for del, respectively. All the somatic mutations were found in the malign patients and located in the coding region, including 6 missense mutations and 5 frameshift mutations.

TLs were significantly shorter in patients with somatic mutations when compared with patients with no mutation in breast tumor tissues (P=0.007). Mean TLs of patients with and without somatic mutation were 0.965 (SE=0.117) and 1.379 (SE=0.039) respectively. The medians and the 25th, and 75th percentiles of TLs in breast tumor patients with and without somatic mutations are shown in Fig. 2A.

Relationship between TL and other common p53 variants. Among the germline variants, four variants were observed at low frequencies [minor allele frequency (MAF) <0.01] in

Table II. Somatic mutations detected in the patients.

Sample name	Telomere length	Type of cancer	Mutation (PCR product)	Genomic description[a]	Exon/intron number	Mutational type	Residue change (Splice site)
5C	1.348	Breast	1856T→C	g.12524	5-exon	A:T→G:C	His179Arg
31C	0.412	Breast	1734G→T	g.12646	6-exon	G:C→T:A	His193Asn
38C	1.009	Breast	1665C→T	g.12715	6-exon	G:C→A:T	Val216Met
39C	0.827	Breast	1941del	g.12443	5-exon	del	Pro152NA
41C	1.559	Breast	2785-2786del	g.11596-11597	4-exon	del	Val122NA
67C	0.958	Breast	2823insCGGA	g.11560	4-exon	ins	Arg110NA
72C	0.688	Breast	547C→T	g.13833	8-exon	G:C→A:T	Glu285Lys
94C	1.371	Breast	1685-1686del	g.12694-12695	6-exon	del	Arg209NA
97C	0.561	Breast	570C→A	g.13810	8-exon	G:C→T:A	Cys277Phe
98C	0.916	Breast	348del	g.14032	9-exon	del	Lys320NA
98C	0.916	Breast	351C→A	g.14029	9-exon	G:C→T:A	Lys319Asn
C085	0.985	Esophageal	1001G→A	g.13379	7-exon	G:C→A:T at CpG	Arg248Trp
C090	0.679	Esophageal	562T→A	g.13818	8-exon	A:T→T:A	Arg280STOP
C091	1.361	Esophageal	582C→T	g.13798	8-exon	G:C→A:T at CpG	Arg273His
C093	0.729	Esophageal	2773C→T	g.11607	4-intron	G:C→A:T	NA (consensus SD)
C094	0.780	Esophageal	582C→G	g.13798	8-exon	G:C→C:G	Arg273Pro
C095	0.880	Esophageal	567G→C	g.13813	8-exon	G:C→C:G	Pro278Arg
C097	2.118	Esophageal	1737G→A	g.12643	6-exon	G:C→A:T	Gln192STOP
C100	0.802	Esophageal	1641C→A	g.12739	6-exon	G:C→T:A	Glu224STOP
C100	0.802	Esophageal	1722C→G	g.12658	6-exon	G:C→C:G	Val197Leu
C101	1.681	Esophageal	1048A→G	g.13332	7-exon	A:T→G:C	Ile232Thr
C102	0.486	Esophageal	1021G→C	g.13359	7-exon	G:C→C:G	Ser241Cys
C104	0.507	Esophageal	1652T→C	g.12728	6-exon	A:T→G:C	Tyr220Cys
C107	0.479	Esophageal	1980C→G	g.12400	5-exon	G:C→C:G	Ala138Pro
C108	1.302	Esophageal	556G→A	g.13824	8-exon	G:C→A:T at CpG	Arg282Trp
C110	0.805	Esophageal	1652T→C	g.12728	6-exon	A:T→G:C	Tyr220Cys
C111	0.714	Esophageal	1652T→G	g.12728	6-exon	A:T→C:G	Tyr220Ser
C112	0.689	Esophageal	982-984del	g.13401-13403	7-exon	del	Ile255NA
C114	1.323	Esophageal	543insTT	g.13838	8-exon	ins	Glu287NA
C114	1.323	Esophageal	583G→A	g.13797	8-exon	G:C→A:T at CpG	Arg273Cys
C115	0.872	Esophageal	1001G→A	g.13379	7-exon	G:C→A:T at CpG	Arg248Trp
C116	1.306	Esophageal	1665C→A	g.12715	6-exon	G:C→T:A	Val216Leu
C120	0.979	Esophageal	2866insA	g.11514	4-exon	ins	Ser95NA
C121	0.481	Esophageal	1990A→C	g.12390	5-exon	A:T→C:G	Phe134Leu
C122	0.526	Esophageal	570C→A	g.13810	8-exon	G:C→T:A	Cys277Phe
C124	0.539	Esophageal	556G→A	g.13824	8-exon	G:C→A:T at CpG	Arg282Trp
C124	0.539	Esophageal	1875C→T	g.12505	5-exon	G:C→A:T	Val173Met
C125	0.916	Esophageal	313A→G	g.14067	9-intron	A:T→G:C	NA (consensus SD)
C127	0.864	Esophageal	561C→A	g.13819	8-exon	G:C→T:A	Arg280Ile
C132	1.890	Esophageal	1865C→A	g.12515	5-exon	G:C→T:A	Cys176Phe
C134	0.432	Esophageal	583G→A	g.13797	8-exon	G:C→A:T at CpG	Arg273Cys
C134	0.432	Esophageal	1737G→A	g.12643	6-exon	G:C→A:T	Gln192STOP
C136	0.528	Esophageal	484G→A	g.13896	8-exon	G:C→A:T at CpG	Arg306STOP
C138	0.562	Esophageal	2955del	g.11425	4-exon	del	Arg65NA
C141	0.410	Esophageal	1868C→T	g.12512	5-exon	G:C→A:T at CpG	Arg175His
C142	1.313	Esophageal	1677-1678del	g.12704-12705	6-exon	del	Phe212NA
C143	0.394	Esophageal	617-637del	g.13743-13763	8-exon	del 7-intron	Ser261NA
C143	0.394	Esophageal	1727A→G	g.12653	6-exon	A:T→G:C	Ile195Thr
C144	0.661	Esophageal	2868G→C	g.11512	4-exon	G:C→C:G	Ser94STOP
C145	0.628	Esophageal	2998C→A	g.11382	4-exon	G:C→T:A	Glu51STOP

Table II. Continued.

Sample name	Telomere length	Type of cancer	Mutation (PCR product)	Genomic description[a]	Exon/intron number	Mutational type	Residue change (Splice site)
C146	0.606	Esophageal	1638C→T	g.12742	6-intron	G:C→A:T	NA (consensus SD)
C146	0.606	Esophageal	2871-2872del	g.11508-11509	4-exon	del	Leu93NA
C147	0.581	Esophageal	576C→A	g.13804	8-exon	G:C→T:A	Cys275Phe
C148	0.794	Esophageal	539C→T	g.13841	8-exon	G:C→A:T	Glu287Glu
C148	0.794	Esophageal	547C→T	g.13833	8-exon	G:C→A:T	Glu285Lys
C148	0.794	Esophageal	666C→T	g.13714	7-intron	G:C→A:T	NA
C149	0.550	Esophageal	568G→A	g.13812	8-exon	G:C→A:T	Pro278Ser
C150	0.369	Esophageal	484G→A	g.13896	8-exon	G:C→A:T at CpG	Arg306STOP
C151	0.514	Esophageal	1941G→A	g.12439	5-exon	G:C→A:T	Pro151Ser
C152	0.919	Esophageal	582C→T	g.13798	8-exon	G:C→A:T at CpG	Arg273His
C153	0.854	Esophageal	583G→A	g.13797	8-exon	G:C→A:T at CpG	Arg273Cys
C155	1.253	Esophageal	1041insG	g.13339	7-exon	ins	Asn235NA
C157	1.096	Esophageal	2877C→T	g.11503	4-exon	G:C→A:T	Trp91STOP
C158	0.738	Esophageal	1033A→T	g.13347	7-exon	A:T→T:A	Met237Lys
C159	0.692	Esophageal	1072T→G	g.13308	6-intron	A:T→C:G	NA (consensus SA)
C160	0.674	Esophageal	618C→T	g.13762	7-intron	G:C→A:T	NA (consensus SA)

[a]Mutation nomenclature according to the HGVS standards with the GenBank NC_000017.9 genomic sequence as reference. NA, not applicable; SD, splice donor site; SA, splice acceptor site.

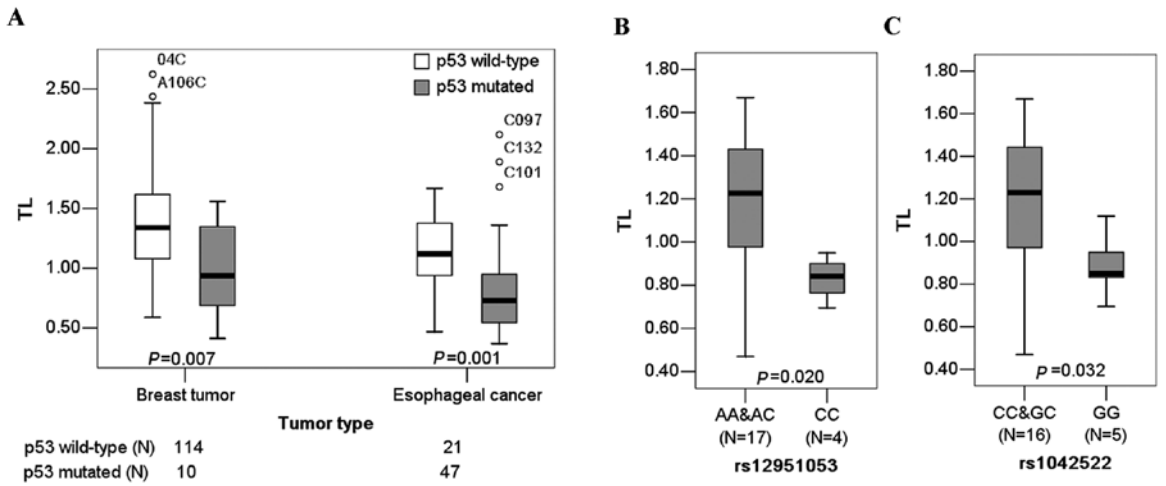

Figure 2. (A) TLs in tumors of breast tumor patients and esophageal cancer patients according to a mutated or wild-type p53 gene. TLs in esophageal cancer tissues according to the (B) rs12951053 and (C) rs1042522 genotypes.

breast tumor patients. Variant 1621 (position in PCR product), which was not included in the known SNPs, was detected in five breast tumor patients both in leukocyte and breast tumor tissue. The remnant common SNPs were rs12951053, rs12947788, rs1625895, rs1042522, rs17883323, rs17878362, rs1642785. The locations of polymorphisms ranged from 2-intron to 7-intron. LD coefficient (D' and r2) and Hardy-Weinberg equilibrium P-value were estimated by Haploview (Table III, Fig. 3A). For complete linkage SNPs, one was selected for subsequent analysis. These were rs12951053, rs1625895, rs1042522, rs17883323 and rs17878362. To obtain accurate results, patients with somatic mutations were excluded of in the association analysis of SNPs, haplotypes and TLs. In the group of breast tumor patients, TLs of the different genotypes did not achieve significant difference for all SNPs in the tumor tissue (Table IV). There was also no significant difference among the TLs of the different haplotypes (data not shown).

Correlation of allelic loss with somatic mutations and TL. In a comparative analysis of *TP53* SNPs in blood and tumor tissues of breast tumor patients, allelic loss was detected in 11.3% (8/71) of tumors from heterozygous patients. Mean TL of patients with allelic loss (1.170, SE=0.173) was shorter than the mean TL of patients with no allelic loss (1.369, SE=0.054)

Table III. Seven common SNPs identified by sequencing.

SNP	Position (PCR product)	Locus	Breast tumor HWE P-value	Breast tumor MAF	Esophageal cancer HWE P-value	Esophageal cancer MAF	Alleles
rs12951053	869	7-intron	0.897	0.381	1	0.353	A:C
rs12947788	889	7-intron	0.897	0.381	1	0.353	G:A
rs1625895	1577	6-intron	1	0.032	1	0.059	C:T
rs1042522	2934	4-exon	0.1973	0.488	0.8499	0.485	C:G
rs17883323	3081	3-intron	0.9608	0.075	0.9501	0.103	G:T
rs17878362	3131	3-intron	1	0.036	1	0.059	-:ccccagcccctccaggt
rs1642785	3263	2-intron	0.1973	0.488	0.8499	0.485	C:G

SNP, single-nucleotide polymorphism; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency.

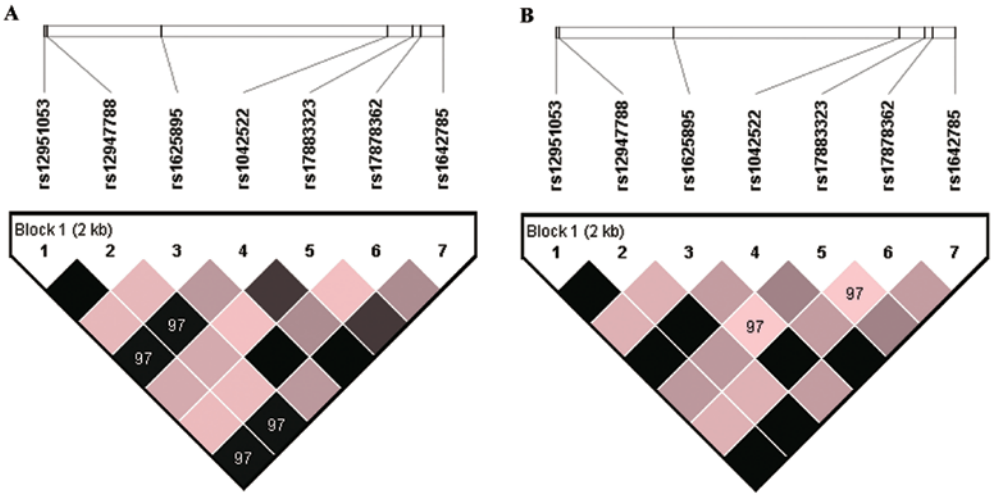

Figure 3. Linkage disequilibrium (LD) plot of 7 common SNPs. The LD plots were constructed using Haploview software. The LD color scheme was D'/LOD(alt). (A) Breast tumor patients; (B) esophageal cancer patients.

with a non-significant P-value (0.178). *TP53* allelic loss was detected in 60.0% (3/5) of breast tumor patients with somatic p53 mutations, which was more in comparison with individuals (7.6%, 5/66) without somatic p53 mutations (P=0.009). This suggests that *TP53* allelic loss was associated with *TP53* mutations among heterozygous breast tumor patients.

In the patients with *TP53* allelic loss, all of the heterozygous *TP53* polymorphisms lost one allele, which suggested that the loss type was large fragment loss. With respect to every polymorphism included, the details of allelic loss are shown in Table V. For the famous rs1042522 (codon 72), 6 of 55 patients heterozygous for codon 72 had allelic loss, including 1 (16.7%) loss of G allele (Pro) and 5 (83.3%) loss of C allele (Arg).

Association of common SNPs and susceptibility to malignant transformation in breast tumor. Associations between common SNPs of *TP53* and the susceptibility to tumor maligning in breast tumors were listed in Table VI. No significant difference was observed between malign and benign tumor patients in 5 common tagSNP genotypes and allele frequencies. This result implies that all of the polymorphisms confer no effect on the risk of tumor maligning in our breast tumor patients. The distribution of haplotypes in benign and malign breast tumor patients were not significantly different (data not shown).

Esophageal cancer

TL and its association with somatic p53 mutations. Of the 68 patients with esophageal cancer investigated in this study, 55 *TP53* gene somatic mutations were found in 47 patients, and 7 patients had more than one mutation. The frequency of *TP53* gene somatic mutations in esophageal cancer was therefore 69.1% (47/68) in our study. All of the 47 patients had at least one mutation causing a amino acid change or located in the splice-site. Among the 55 somatic mutations identified, there were 31 missense mutations, 9 nonsense mutations, 8 frameshift mutations, 5 splice-site mutations, 1 silent mutation and 1 intronic mutation. The proportions of different mutational types were 5/55 (9.1%) for A:T→G:C, 12/55 (21.8%) for G:C→A:T at CpG, 12/55 (21.8%) for G:C→A:T, 2/55 (3.6%) for A:T→T:A, 6/55 (10.9%) for G:C→C:G, 7/55 (12.7%) for G:C→T:A,

Table IV. Associations of p53 common variants in tumors without p53 somatic mutations and TLs in breast tumors and esophageal cancer.

	Breast tumor		Esophageal cancer	
Genotype	N	TL (means ± SE)	N	TL (means ± SE)
rs12951053				
AA	43	1.356±0.062	10	1.221±0.109
AC	52	1.376±0.057	7	1.139±0.105
CC	19	1.441±0.109	4	0.832±0.052
P-value		0.958		0.052
rs1625895				
CC	106	1.366±0.039	20	1.108±0.071
CT	8	1.551±0.229	1	1.351
P-value		0.606		0.509
rs1042522				
CC	33	1.377±0.064	7	1.106±0.129
GC	49	1.360±0.060	9	1.258±0.103
GG	32	1.411±0.085	5	0.889±0.070
P-value		0.962		0.079
rs17883323				
GG	99	1.408±0.042	18	1.071±0.071
GT	15	1.187±0.094	3	1.414±0.160
P-value		0.108		0.088
rs17878362				
-/-	105	1.361±0.039	20	1.108±0.071
-/ccccagccctccaggt	9	1.591±0.206	1	1.351
P-value		0.344		0.509

3/55 (5.5%) for A:T→C:G, 3/55 (5.5%) for ins and 5/55 (9.1%) for del, respectively. Transitions were predominant (29/55, 52.7%), followed by transversions (18/55, 32.7%).

In 68 esophageal cancer samples, TL was determined by real-time PCR. The mean level of TL in esophageal cancer tissues was 0.923 (SE=0.047). TLs were plotted against patient age at sampling (Fig. 1B). No correlation was found between age and TL in esophageal cancer tissue. TLs were significantly shorter in patients with somatic mutations compared with patients with no mutation in esophageal cancer tissues (P=0.001). Mean TLs of patients with and without somatic mutations were 0.835 (SE=0.057) and 1.120 (SE=0.069), respectively. The medians, the 25th and the 75th percentiles of TLs in the esophageal cancer patients with and without somatic mutations are shown in Fig. 2A.

Relationship between TL and other common p53 variants. Among the germline variants, two variants were observed at low frequencies (MAF <0.01) in esophageal cancer patients. The remnant common SNPs were rs12951053, rs12947788, rs1625895, rs1042522, rs17883323, rs17878362 and rs1642785. Linkage disequilibrium coefficient (D' and r2) and Hardy-Weinberg equilibrium P-value were estimated by Haploview (Table III, Fig. 3B). The SNPs selected for subsequent analysis were the same as for the breast tumors.

In the group of esophageal cancer patients, TLs of patients with minor genotype CC of rs12951053 and GG of rs1042522 were significantly shorter than patients with other genotypes of this SNP in esophageal cancer tissue. Mean TLs of patients with genotypes CC and AA&AC of rs12951053 were 0.832 (SE=0.052) and 1.187 (SE=0.076) respectively (P=0.020). Mean TLs of patients with genotypes GG and CC&GC of rs1042522 were 0.889 (SE=0.070) and 1.192 (SE=0.080), respectively (P=0.032). The medians, the 25th and the 75th percentiles of TLs in the esophageal cancer tissues according to genotypes of rs12951053 and rs1042522 are shown in Fig. 2B and C. For other SNPs, TLs of different genotypes did not achieve significant difference (Table IV). Haplotypes of 7 common SNPs were estimated using the Phase software. Patients with haplotype CACGG-G (rs12951053, rs12947788, rs1625895, rs1042522, rs17883323, rs17878362, rs1642785) had a significantly shorter TL than patients with the other haplotypes (P=0.009). Mean TLs of patients with haplotype CACGG-G and the other haplotypes were 0.975 (SE=0.065) and 1.200 (SE=0.061), respectively.

Correlation of allelic loss with somatic mutations and TL. In a comparative analysis of *TP53* SNPs in tumor and normal tissue of esophageal cancer patients, allelic loss was detected in 57.8% (26/45) of tumors from heterozygous patients. The

Table V. Details of the patients with allelic loss.

Patient no.	Type of tumor	LOH type	Genotype: blood/normal tissue→tumor							*TP53* mutation
			rs12951053	rs12947788	rs1625895	rs1042522	rs17883323	rs17878362	rs1642785	
20	Breast cancer	Partial loss	AC→A-	AG→G-	-	-	GT→T-	-	-	-
38	Breast cancer	Partial loss	-	-	-	GC→G-	GT→T-	-	GC→G-	+
64	Breast cancer	Partial loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	-
67	Breast cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
94	Breast cancer	Partial loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
A18	Breast tumor	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	-
A71	Breast tumor	Complete loss	AC→C-	AG→A-	-	-	GT→G-	-	-	-
A98	Breast tumor	Partial loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	-
85	Esophageal cancer	Complete loss	-	-	CT→C-	-	GT→T-	A1A2→A1-	-	+
90	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
91	Esophageal cancer	Complete loss	AC→A-	AG→G-	CT→T-	-	-	A1A2→A2-	-	+
93	Esophageal cancer	Partial loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
94	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
95	Esophageal cancer	Complete loss	-	-	-	GC→C-	GT→G-	-	GC→C-	+
100	Esophageal cancer	Complete loss	-	-	-	GC→G-	GT→T-	-	GC→G-	+
101	Esophageal cancer	Complete loss	-	-	-	GC→C-	GT→G-	-	GC→C-	+
102	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
104	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
107	Esophageal cancer	Complete loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
111	Esophageal cancer	Complete loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
112	Esophageal cancer	Partial loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
115	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
116	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
127	Esophageal cancer	Partial loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
136	Esophageal cancer	Partial loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
141	Esophageal cancer	Complete loss	AC→A-	AG→G-	-	-	GT→T-	-	-	+
142	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	-	GT→G-	-	-	+
144	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
145	Esophageal cancer	Partial loss	AC→A-	AG→G-	CT→T-	-	-	A1A2→A2-	-	+
150	Esophageal cancer	Complete loss	AC→A-	AG→G-	CT→T-	-	-	A1A2→A2-	-	+
151	Esophageal cancer	Complete loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+
153	Esophageal cancer	Complete loss	AC→C-	AG→A-	-	GC→G-	-	-	GC→G-	+
158	Esophageal cancer	Complete loss	-	-	-	GC→G-	GT→T-	-	GC→G-	+
160	Esophageal cancer	Complete loss	AC→A-	AG→G-	-	GC→C-	-	-	GC→C-	+

A1, non-duplicated allele; A2, duplicated allele; -, no LOH and *TP53* mutation; +, presence of *TP53* mutation.

Table VI. Genotype frequencies of common SNPs and their association with susceptibility to malignant transformation in breast tumors.

SNP	Group	Genotype frequency n, (%)		
		AA	AC	CC
rs12951053	Benign	27 (33.3)	43 (53.1)	11 (13.6)
	Malign	22 (48.9)	15 (33.3)	8 (17.8)
P-value				0.101
		CC	CT	
rs1625895	Benign	77 (95.1)	4 (4.9)	
	Malign	41 (91.1)	4 (8.9)	
P-value				0.455
		CC	CG	GG
rs1042522	Benign	22 (27.2)	38 (46.9)	21 (25.9)
	Malign	15 (33.3)	17 (37.8)	13 (28.9)
P-value				0.600
		GG	GT	
rs17883323	Benign	70 (86.4)	11 (13.6)	
	Malign	37 (82.2)	8 (17.8)	
P-value				0.528
		-/-	-/ccccagccctccaggt	
rs17878362	Benign	76 (93.8)	5 (6.2)	
	Malign	41 (91.1)	4 (8.9)	
P-value				0.720

SNP, single-nucleotide polymorphism.

mean TL of patients with allelic loss (0.789, SE=0.063) was shorter than the mean TL of patients with no allelic loss (1.008, SE=0.092) with borderline statistical significance (P=0.056). The frequency of *TP53* allelic loss was significantly higher in heterozygous esophageal cancer patients with somatic mutations compared with patients with no mutation (P<0.001). The frequencies of *TP53* allelic loss were 74.3% (26/35) for mutation esophageal tumors and 0.00% (0/10) for no-mutation esophageal tumors. These suggest that *TP53* allelic loss was associated with *TP53* mutations among heterozygous esophageal cancer patients.

In the patients with *TP53* allelic loss, all of the heterozygous *TP53* polymorphisms lost one allele which was the same as for breast tumors (Table V). For the famous rs1042522 (codon 72), 20 of 36 patients heterozygous for codon 72 had allelic loss, including 9 (45.0%) loss of G allele (Pro) and 11 (55.0%) loss of C allele (Arg).

Discussion

The most important function of telomeres is the maintenance of genomic integrity and stability (1,2). TL of human somatic cells is a biomarker of cumulative oxidative stress, biologic age and life stress (19,20). It shortens with each cell division (4,5). Oxidative stress (21) and life stress (22) also can accelerate its shortening. We observed an inverse correlation between TL and age in breast tumor tissue, demonstrating a significant age-related telomere loss in these tissues. This correlation was not detected in esophageal cancer tissue, which suggested that other factors, such as oxidative stress and life stress, rather than age mainly influence the TL in these tissues.

Mutation of the tumor suppressor p53 is an almost universal feature of human cancer. In our present study, we detected 11 somatic mutations in 10 breast tumor patients. All of the mutations were located in the coding region and caused amino acid changes. G:C→A:T mutations are very frequent in sporadic breast cancers (IARC *TP53* database, http://www-p53.iarc.fr, R15 release) (11). Compared with global *TP53* mutations in sporadic breast cancer, mutations in our patients had less G:C→A:T transitions (18.18 vs. 46.56%) and more deletions (36.36 vs. 11.17%) and G:C→T:A transversions (27.27 vs. 8.90%). Of the 68 patients with esophageal cancer investigated in this study, 55 *TP53* gene somatic mutations were found in 47 patients. The proportions of different mutational types in our esophageal cancer patients are similar with global *TP53*

mutation in esophageal cancer (11), with a higher transition followed by transversion.

Previous research has shown that mutant p53 proteins have a dominant negative effect on wild-type p53, and inhibit or activate the function of other p53 family members (13). Inhibition of p53 function enables continuous cell division and critical telomere shortening, a phenomenon known as telomere crisis, which causes telomere fusion and genome instability (3,10,23). Our study showed that telomeres were statistically shorter in tumor/cancer tissue from patients with *TP53* somatic mutations than those with wild-type. This finding suggests that mutant p53 enables continuous cell division and critical telomere shortening and combines telomere erosion driving tumor formation.

Chromosomal instability (CIN) is a feature of most human cancers, and one mechanism of CIN is though the loss of telomeres (24). LOH is one of the representations of chromosomal instability, and short telomeres have been reported to contribute to LOH in renal cell carcinoma (25). In our study, patients with allelic loss had a shorter TL than patients with no allelic loss, and *TP53* allelic loss was associated with *TP53* mutations among heterozygous patients in both tumor types. These results suggest that large fragment *TP53* allelic loss may be one of the representations of chromosomal instability caused by telomere dysfunction combined with p53 function inhibition. Notably, the patients with p53 allelic loss had a high proportion of mutant alleles (50-100%). LOH has emerged as the second hit in tumor initiation which serves to inactivate or eliminate the wild-type allele at the tumor-suppressor gene locus (12,15). Thus, LOH at the p53 locus caused by chromosomal instability may constitute one of the major mechanisms for inactivation of the intact allele associated with a p53 mutation (16).

Combined with our results and previous studies, we hypothesize that the mechanisms of tumorigenesis associated with telomere dysfunction and p53 mutations are as follows. i) Telomere DNA is progressively lost with each cell division (4,5). ii) Telomere shortening reaching a critically short length activates DNA damage checkpoints, and results in induction of cellular senescence, and the first checkpoint in response to telomere shortening is a p53-dependent, permanent cell cycle arrest (6). iii) Exogenous carcinogens and endogenous biological processes cause p53 mutations (26). iv) Mutant p53 proteins enable continuous cell division and critical telomere shortening, a phenomenon known as telomere crisis, which causes telomere fusion and genome instability. v) LOH occurring by chromosomal instability inactivates the intact allele associated with p53 mutation. vi) Recurrence of the above steps occurs. vii) Tumorigenesis and malignant transformation transpires.

Rs1042522, viz. codon R72P SNP, is in exon 4, the segment of *TP53* that encodes the polyproline domain, which is essential for p53 to mount a full apoptotic response to stress and inhibit tumorigenesis (14). It has been reported that p53-P72 has a weaker apoptotic potential than p53-R72 (27). In esophageal cancer tissue, we detected that patients with a minor genotype GG of rs1042522 had a shorter TL than those with genotypes CC&GC, and patients with minor genotype CC of rs12951053 had a shorter TL than those with genotypes AA&AC. Genotype GG and CC of rs1042522 were corresponded to P72 and R72 in our study. Thus, the minor genotype GG has a weaker apoptotic potential, and may enable critical telomere shortening. Rs12951053 is in intron 7, which has a strong linkage relationship with rs1042522 (Fig. 3). Its significant difference in TL between genotypes may be caused by this. Patients with haplotype CACGG-G have a significantly shorter TL than patients with the other haplotypes. This haplotype exclusively contains C allele of rs12951053 and G allele of rs1042522 simultaneously. The above-mentioned differences did not exist in breast tumor patients, which suggests that the function of SNPs may be tissue- or tumor type-specific. For other SNPs, we found no evidence for an association with TL. Their TLs of different genotypes did not show a significant difference. Our results showed that SNPs of *TP53* may, depending on tissue or tumor type, specifically have a very feeble effect on cellular senescence and/or apoptosis associated with telomere dysfunction. Elucidation of this issue requires investigation with a large sample size and the use of more types of cancer.

Although the relationships between *TP53* variants and TL in breast tumor and esophageal cancer have not been directly studied previously, several similar studies exist. Two studies found that TLs in the peripheral blood cells of germline p53 mutation carriers of Li-Fraumeni syndrome were shorter than that of normal individuals (28,29). Another similar research by Radpour *et al* found that TL is inversely correlated with the promoter methylation profile of p53 in breast cancer, which suggests that p53 may function as a gatekeeper to prevent critical telomere shortening and genome instability (30). From the above findings, it is evident that all similar research obtained consistent results consistent with ours suggesting that TL shortening cannot drive tumorigenesis alone; it is combined with defects in cellular senescence and/or apoptosis. p53 plays a key role in this pathway. This may explain the inconsistent results of previous research investigating TL and cancer risk (31-34). Thus, in future research concerning telomere dysfunction and cancer risk, the effects of cellular senescence and apoptosis should also be considered.

In conclusion, our study revealed that telomeres of patients with *TP53* somatic mutations were statistically shorter than those with wild-type in both breast tumor tissue and esophageal cancer tissue, and large fragment *TP53* allelic loss was significantly associated with somatic mutations. These findings suggest that mutant p53 enables continuous cell division and critical telomere shortening and combines telomere erosion driving tumor formation. Large fragment *TP53* allelic loss may be one of the representations of chromosomal instability caused by telomere dysfunction combined with p53 function inhibition. The SNPs of *TP53* depending on tissue or tumor-type may have a feeble effect on cellular senescence and/or apoptosis associated with telomere dysfunction. Investigation with a large sample size using more types of cancers may elucidate this issue.

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