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Shortened Telomeres in Serous Tubal Intraepithelial Carcinoma: An Early Event in Ovarian High-grade Serous Carcinogenesis

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Abstract

Short telomeres are one of the main genetic manifestations in human cancer, as they have been shown to play an important role in inducing chromosomal instability and in contributing to tumor progression. The purpose of this study was to determine if changes in telomere length occur in serous tubal intraepithelial carcinoma (STIC), the putative precursor of “ovarian” high-grade serous carcinoma (HGSC). Twenty-two STICs from 15 patients with concurrent but discrete HGSCs were analyzed for telomere length on formalin-fixed, paraffin-embedded sections by conducting p53 immunofluorescence to assist in identifying STICs and telomere-specific FISH. Telomere length (short, long, or no change) in STICs was compared with HGSCs using normal fallopian tube epithelium and stromal cells as controls. We found that STICs had the shortest telomeres, as 18 (82%) of 22 STICs had short telomeres, whereas only 2 (9%) showed no change and 2 (9%) had long telomeres compared with the normal-looking tubal epithelium. In contrast, among 12 paired HGSCs and STICs, 6 HGSCs showed an increase in telomere length, one showed a decrease in length and 5 did not show any change when compared with their matched STICs, although, such as STICs, the majority of HGSCs had shorter telomeres than the associated normal tubal epithelial cells. These differences in telomere length between normal tubal epithelial cells and STICs, and between STICs and HGSCs were statistically significant ($P < 0.05$). In conclusion, the finding of short telomeres, which have been shown to be one of the earliest molecular changes in carcinogenesis, in a vast majority of STICs provides further support to the proposal that STICs are precursors of HGSC and opens new areas of research in elucidating the early events of ovarian high-grade serous carcinogenesis.

Keywords

ovarian cancer; serous tubal intraepithelial carcinoma; telomere; high-grade serous carcinoma

Telomeres are DNA sequences composed of short tandem repeats located at the ends of chromosomes that protect them from aberrant fusion or degradation. Attrition of telomeres occurs after each cell division in normal cells because DNA polymerases are not able to fully replicate chromosomal ends. Activation of the DNA damage response pathway, which leads to cellular senescence or apoptosis, occurs if telomeres shorten below a critical length.^{40,44} It has been proposed that telomere attrition has evolved as a tumor suppression mechanism³ based on studies of mouse tumor models.^{7,13} In germ cells, certain stem cells, and highly proliferative somatic cells, telomere length is largely maintained as a result of complex regulatory mechanisms including the upregulation of telomerase, a polymerase that uses RNA as a template.⁴ Telomere integrity in these cells is essential to support their extended replicative potential and similarly most human cancers upregulate telomerase expression. In general, tumors compared with the normal tissues from which they are derived, have shorter telomeres probably because active cell division occurs in the tumor initiation stage before telomerase gene upregulation. Accordingly, telomere length has been used as a biomarker in predicting cancer risk and prognosis.⁴¹

Ovarian high-grade serous carcinoma (HGSC) is the most lethal gynecologic malignancy and is characterized by frequent *TP53* mutations^{6,36} and a high level of chromosomal instability that is reflected by widespread DNA copy number changes compared with other types of epithelial ovarian carcinomas.²⁰ Unlike cancers arising in the colon, breast, cervix, endometrium, prostate, and pancreas, for which precursor lesions have been well recognized, serous tubal intraepithelial carcinomas (STICs) have only recently been identified as putative precursors of HGSC based on studies showing identical *TP53* mutations and similar high levels of chromosomal instability in both lesions.^{9,19,33,35,37} Additional evidence supporting this proposal is the observation that STICs are more frequently detected in the fallopian tubes of women with hereditary BRCA mutations than in the tubes of women without ovarian or tubal carcinoma.³⁹ Recently, we showed that STICs express several potential onco-genes frequently found in HGSC,³⁸ further linking both lesions. In addition, gene expression profiling has showed that HGSCs from the fallopian tube and ovary are indistinguishable.⁴² Finally, the fact that both more closely resemble fallopian tube epithelium than ovarian surface epithelium,²⁵ which has been long thought as the cell of origin of “ovarian” HGSC. Taken together, both morphologic and molecular studies have suggested the tubal origin of most ovarian HGSCs.²¹

Telomerase activity and shorter telomeres have been found in ovarian cancer tissue, with the highest telomerase activity detected in carcinoma among cyst, borderline tumor and normal ovarian tissues.^{8,10,30,34,45} Given the fundamental role of telomeres in carcinogenesis, we undertook an analysis of telomere length in STICs and HGSCs in an attempt to verify whether telomere shortening occurs in STICs thereby providing further evidence that they are precursors of HGSC and to further elucidate the molecular changes in early ovarian serous carcinogenesis.

MATERIALS AND METHODS

Case selection

Twenty-two STICs from 15 patients who had concurrent stage IIIC/IV ovarian/pelvic HGSC were selected for analysis of telomere length. Among them, 12 specimens contained STICs and HGSC in the same block. This set was compared with 134 HGSC (all stage IIIC/IV) that served as a validation set. All the cases were retrieved from the surgical pathology files in the Department of Pathology at the Johns Hopkins Hospital. Many of the STIC specimens were sent as consultation cases from the Legacy Health Systems, Portland, Oregon. Tissue collection conformed to the guidelines of the Institutional Research Board of the Johns Hopkins Hospital. All STICs were contiguous to benign tubal epithelium but were discrete from the invasive carcinomas. The morphologic criteria used to define a STIC were modified from a earlier report.²³ To qualify as a STIC, the lesion had to be composed of nonciliated cells exhibiting 3 or more of subsequent features: (1) abnormal chromatin pattern, (2) nuclear enlargement, (3) marked nuclear pleomorphism, (4) epithelial stratification and/or loss of polarity, and (5) nuclear molding.

p53 Immunostaining and Telomere Fluorescence in Situ Hybridization

To facilitate identification of STICs, immunofluorescence was carried out using an anti-p53 antibody, in combination with telomere length scoring by in situ hybridization fluorescence (FISH) using a earlier described technique.²⁷ In brief, after deparaffinization and hydration, slides were placed in citrate buffer (Vector Laboratories, Burlingame, CA) for antigen retrieval. Cy3-labeled telomere-specific peptide nucleic acid (PNA) (Boehringer-Mannheim, Indianapolis, IN) was applied to the sample, which was then coverslipped, and incubated at 83°C for 4 minutes. The specimens were then hybridized in a closed chamber at room temperature for 2 hours. The coverslips were removed, the slides washed twice in a PNA wash solution (70% formamide, 10 mmol/L Tris, pH 7.5, 0.1% albumin) followed by PBST and then incubated with an anti-p53 antibody (antibody DO1, Dako Cytomation) at a dilution of 1:100 at 4°C overnight. A fluorescently labeled secondary antibody [goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes Cat.# A-11001)] was then applied at a dilution of 1:100 in PBS, and the slides incubated at room temperature for 30 minutes after which they were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) at a concentration of 500 ng/mL (Sigma) for 3 minute at room temperature, washed in water, mounted with Prolong anti-fade mounting medium (Molecular Probes Inc., Eugene, OR), and imaged. The PNA probe, complementary to the mammalian telomere repeat sequence, was obtained from Applied Biosystems (Framingham, MA), and contained the sequence N-terminus to C-terminus CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. A FITC-labeled PNA probe with the sequence ATTCGTTGGAAACGGGA that is specific for human centromeric DNA repeats (CENP-B binding sequence) was included in the hybridization solution⁵ as a positive control of hybridization efficiency.

Microscopy and Image Analysis

To measure the relative changes in telomere length, the slides were imaged with a Nikon 50i epifluorescence microscope equipped with an X-Cite series 120 illuminator (EXFO

Photonics Solutions Inc., Ontario, CA) and a $100\times/1.4$ NA oil immersion Neofluar lens. Fluorescence excitation/emission filters were as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm through an XF02 fluorescence set (Omega Optical, Brattleboro, VT); Alexa Fluor 488 excitation, 475 nm; emission, 535 nm through a combination of 475RDF40 and 535RDF45 filters (Omega Optical). Images were captured using the Photometrics CoolsnapEZ digital camera equipped with the Nikon NIS-Elements software. Quantitative FISH was conducted on 12-bit grayscale images of the tissue sections as described²⁷ using a custom plug in (<http://bui2.win.ad.jhu.edu/telometer/>) specific to the program ImageJ. The telomere fluorescence intensity was calculated as the ratio of total Cy3 intensity to the total DAPI intensity for each sampled nucleus to correct for any ploidy differences or nuclear cutting artifacts. At least 30 nuclei were analyzed for each tissue component.

Immunohistochemistry

The MIB-1 antibody to Ki-67 (Ventana, Tucson, AZ; 1:1 dilution) and γ H2AX antibody (Cell Signaling, Danvers, MA, 1:200 dilution) were used in immunohistochemical staining of formalin-fixed paraffin sections. Antigen retrieval was carried out by steaming the sections in citrate buffer (pH 6.0) for 20 min. After incubation with the primary antibodies at room temperature for 2 hrs, a positive reaction in tissue sections was developed with 3,3'-diaminobenzidine and detected with the EnVision + System (DAKO, Carpinteria, CA). The percentage of intensely immunoreactive nuclei was determined by 2 pathologists (EK and IMS). In the process of cutting sections for immunohistochemistry, 4 of the STICs were exhausted and therefore they were not evaluated for γ H2AX antibody.

Statistical Analysis

We correlated telomere length between STICs and HGSCs and compared their telomere lengths to normal tubal epithelium and stromal cells from the same specimens using a paired *t* test (Mann-Whitney test). Linear regression was used to determine the correlation of telomere length between the normal tubal epithelium and the lesions, between telomere length and the Ki-67 proliferation index (percentage of Ki-67 positive cells) and between telomere length and percentage of γ H2AX-labeled STIC cells.

RESULTS

Of the 22 STICs, 18 (81.8%) were flat lesions and 4 (18.2%) were papillary (T1, T8, T11, and T19) and although most were solitary, they were multiple in 6 patients (Fig. 1 and Table 1). Fifteen (68.2%) STICs were located in the fimbriated end of the fallopian tubes, and were present in the same tissue sections that contained invasive carcinomas in the ovary or peritubal soft tissue and as earlier reported were morphologically similar to the associated HGSCs.

The relative telomere length (mean and standard deviation) for STICs, HGSCs, normal-looking tubal epithelium and stromal cells is shown in Figure 2A and representative examples of telomeres using FISH and p53 immunofluorescence are shown in Figure 2B. The longest telomeres were in stromal cells followed by normal tubal epithelium, HGSCs

and STICs. The most significant finding was a decrease in telomere length in STICs compared with adjacent normal tubal epithelium ($P < 0.005$) as 18 had statistically significant shorter telomeres than those in the matched normal tubal epithelium whereas 2 STICs had longer telomeres (Fig. 3). The telomeres in the remaining 2 STICs showed no significant change compared with normal tubal epithelium from the same patients (Fig. 3A). Moreover, all individual STICs in multifocal STICs from the same patients showed the same pattern of telomere length change. Using a paired comparison, we found a highly significant difference in overall telomere length between STICs and the corresponding normal tubal epithelium ($P < 0.0001$). Furthermore, among 15 STICs and concomitant HGSCs, 6 HGSCs showed a statistically significant ($P < 0.005$) increase in telomere length compared with STICs whereas only one HGSC exhibited significantly shorter telomeres (Fig. 3A). The remaining 5 HGSCs did not show any significant alteration in telomere length. In summary, 18 (82%) of 22 STICs and 8 (67%) of 12 HGSCs showed shorter telomeres compared with normal tubal epithelium in the same specimen whereas 2 (9.1%) of 22 STICs and 3 (25%) of 12 HGSCs had longer telomeres compared with normal tubal epithelium (Table 1 and Fig. 3B). To determine how the 12 HGSCs that were associated with STICs compared with conventional advanced stage ovarian HGSCs we conducted telomere FISH analysis on 134 advanced stage HGSCs that were arranged in tissue microarrays. There was no statistical significant difference in telomere length between the set of 12 HGSCs and the set of 134 HGSCs ($P = 0.932$) (Fig. 4).

There was a significant direct correlation of telomere length between STICs and HGSCs ($R^2 = 0.573$) and normal tubal epithelium and tubal stromal cells ($R^2 = 0.437$) (Fig. 5). In contrast, there was no significant correlation in telomere length between STICs and normal tubal epithelium ($R^2 = 0.0531$) and between STICs and stromal cells from the same specimens ($R^2 = 0.00501$). There was also no correlation between telomere length in STICs and the Ki-67 proliferation index ($R^2 = 0.0022$) and only a weak correlation between telomere length in STICs and the percentage of γ H2AX positive cells ($R^2 = 0.1021$) (Table 1).

DISCUSSION

The data presented here suggest that measurable somatic telomere length change does occur in STIC, the putative precursor of HGSC. We found shortened telomeres in the majority of STICs compared with adjacent normal tubal epithelium that is consistent with other reports showing telomere shortening in preneoplastic cells in prostate, pancreatic, breast, cervical, colon, lung, and biliary tract carcinomas.^{16,18,26,28} Moreover, the majority of HGSCs showed longer telomeres compared with STICs, although a majority were shorter than normal tubal epithelium. These findings argue against STICs being metastases from an ovarian carcinoma as if that were the case, both lesions would have telomeres of similar length. Our results therefore, lend further support to the hypothesis that STICs are precursors of ovarian HGSC. The changes in telomere length between the preinvasive and invasive carcinomas suggest that stabilization of telomeres is essential in supporting tumor progression as short telomeres are not capable of maintaining the high level of cell division that characterize most carcinomas.¹⁵ The underlying molecular mechanisms that stabilize telomere length are complicated and probably include upregulation of telomerase although

other mechanisms involved in cell cycle regulation are undoubtedly involved^{14,29} because no correlation between the telomerase activity and telomere length was reported in ovarian tumor tissues.⁴⁵

It has been reported that one possible mechanism leading to telomere shortening is oxidative stress that results in cellular damage.⁴³ This is an intriguing hypothesis because reactive oxidant species (free radicals) are released from antrum fluid at the time of ovulation^{31,32} and ovulation has been long regarded as playing a critical role in high-grade ovarian serous carcinogenesis. In fact, it is thought that *BRCA1* expression has evolved to protect tubal epithelium from ovulation-induced DNA damage as *BRCA1* deficient cells have been shown to exhibit chromosomal aberrations and hypersensitivity to oxidative stress.¹ Moreover, *BRCA1* mutation carriers have increased 8-oxo-dG, a surrogate DNA damage marker, in leukocyte DNA.¹² It would be of considerable interest to determine when alteration of telomere length occurs given the complex BRCA and p53 pathways involved in tumor initiation.³⁹

Telomere attrition resembles DNA strand breaks, which through the formation of dicentric chromosomes by end-to-end fusion, results in chromosome breakage and rearrangement that then eventually in chromosomal instability.²² Accordingly, short telomeres may trigger a DNA damage response and induce a p53-dependent growth arrest or cellular senescence in the epithelial cells of a STIC that harbors mutated *TP53* in most cases, thereby allowing epithelial cells with abnormally short telomeres to evade DNA damage-induced, p53-dependent cellular senescence or apoptosis. Thus, telomere shortening and mutant p53 cooperate in promoting the development of carcinoma.¹⁷ Support for this hypothesis is the presence of γ H2AX DNA damage in the nuclei of most STICs as shown in this and another study,²⁴ the demonstration of chromosomal instability in STICs based on FISH analysis of centromere number in selected chromosomes and the high frequency of *TP53* mutations in STICs and HGSCs.^{19,37} The lack of a strong correlation between γ H2AX and telomere length suggests that several other mechanisms such as oncogenic stress, that is induction of oncogenes such as *c-myc* and *Ras* leads to senescence or apoptosis, also play a role in contributing to DNA damage. It is plausible that such unchecked DNA damage is responsible for creating a repertoire of subclones in STICs in which cells that are capable of dissemination and growth are selected for propagation, as suggested by the observation that, short telomeres contribute to the initiation of type II endometrial carcinomas but not type I endometrial carcinomas.² Type II endometrial carcinomas, which are almost always HGSC, have similar morphologic features and other characteristics of ovarian HGSC including aggressive behavior, frequent *TP53* mutations and high levels of chromosomal instability.¹¹

In conclusion, our analysis of telomere length in STICs, HGSCs and normal tubal epithelium suggests that during the development of HGSC, telomeres in tubal epithelium become shortened possibly owing to ovulation-induced oxidative stress, which in turn contributes to chromosomal instability and then to the development of a STIC. Progression from a STIC to a HGSC may occur in those STICs that escape telomere-induced senescence and have acquired additional molecular changes that maintain telomere length. This finding underscores the role of stabilization of telomeres in tumor progression and provides new insight into the early molecular events of ovarian HGSC that may have important clinical

implications. For example, vitamin E and progesterone have been shown experimentally to reduce ovulation-induced oxidative stress and enhance the repair capacity of ovarian surface epithelial cells.³¹ Also epidemiologic studies have provided cogent evidence that a reduction in the number of ovulations in a woman's life, either from the use of oral contraceptives or as a result of pregnancy/lactation, is one of the most important factors in reducing the risk of ovarian cancer. Accordingly, future studies should attempt to assess the efficacy of these, or similar agents, in reducing the risk of ovarian cancer using STICs as surrogate markers for invasive HGSCs given the mounting evidence implicating STICs as the precursor lesion of ovarian HGSC.

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FIGURE 1.

Examples of serous tubal intraepithelial carcinoma (STIC). A, Hematoxylin and eosin stained section in a STIC with flat architecture. B, The same case that was stained with a p53 antibody shows diffuse and intense p53 immunoreactivity. C, Hematoxylin and eosin stained section in a STIC with papillary architecture. D, The same case that was stained with a p53 antibody shows diffuse and intense p53 immunoreactivity.

FIGURE 2.

Relative telomere length in normal-looking fallopian tube epithelium (NFT), serous tubal intraepithelial carcinoma (STIC), high-grade serous carcinoma (HGSC) and tubal stromal cells. A, Telomeres are significantly shorter in STICs as compared with normal fallopian tube epithelium ($P = 0.0037$) and are longer in HGSCs compared to STICs ($P = 0.038$). B, Telomere hybridization signals measured by FISH using p53 immunofluorescence to highlight and distinguish the STICs from the adjacent normal-looking tubal epithelium in 2 representative STICs (STIC-A and STIC-B). The telomere hybridization signals (red fluorescence in the bottom panel) are weaker in the p53 positive STIC (green nuclear fluorescence in the top panel) compared with the normal-looking adjacent tubal epithelium and stromal cells. The tissue sections were counterstained with DAPI.

FIGURE 3.

Paired comparison of telomere length between normal-looking fallopian tube epithelium (NFT) and serous tubal intraepithelial carcinoma (STIC), and between STIC and high-grade serous carcinoma (HGSC) from the same patients. A, Scatter plot shows the telomere length in NFT, STIC and HGSC from the same case. The lines connect each lesion from the same cases to indicate the change in telomere length in individual patients. B, The summary of the percentage of cases showing short telomeres, long telomeres and those showing no change.

FIGURE 4.

Scatter plot analysis of the relative telomere length in HGSCs from the 12 cases with STICs compared with an independent set of 134 conventional high-grade, advanced stage ovarian serous carcinomas. There is no significant difference between the 2 groups ($P = 0.932$).

FIGURE 5.

Correlation of telomere length between groups of normal-looking fallopian tube epithelium, serous tubal intraepithelial carcinoma (STIC), high-grade serous carcinoma (HGSC) and stromal cells. Linear regression (R^2) was used to analyze the correlation between the 2 groups. Each dot represents an individual specimen. A, HGSCs compared with STICs. B, Stromal cells compared with normal-looking tubal epithelium. C, STICs compared with normal-looking tubal epithelium. D, Stromal cells compared with STICs.

TABLE 1
The Relative Telomere Length in Normal-looking Fallopian Tube (NFT), Serous Tubal Intraepithelial Carcinoma (STIC) and High-grade Serous Carcinoma (HGSC) in the Same Patients

Patient No.	STIC No.	NFT			STIC			HGSC		P-value STIC Compared With NFT	P-value STIC Compared With HGSC	P-value HGSC Compared With NFT	Ki67 (%)	γH2AX (%)	p53 (%) [*]
		Mean	SD	Mean	SD	Mean	SD	Mean	SD						
1	T1	9.9	3.7	6.5	19.7	1.5	1.7			0.3567	0.174	<0.0001	90	0	100
2	T2	8.6	5.9	4.3	2.6	4.0	3.6			0.0006	0.713	0.0006	60	50	100
2	T3	8.6	5.9	4.9	3.7	4.0	3.6			0.0051	0.344	0.0006	40	60	90
3	T4	17.5	5.1	3.3	2.5	NA	NA			<0.0001	NA	NA	5	0	Faint
3	T5	17.5	5.1	6.8	3.3	NA	NA			<0.0001	NA	NA	20	0	Faint
4	T6	22.0	7.8	7.0	4.5	14.2	6.6			<0.0001	0.0001	<0.0001	60	100	100
4	T7	22.0	7.8	3.0	2.1	14.2	6.6			<0.0001	0.0001	<0.0001	60	100	0
5	T8	9.2	4.4	3.9	4.6	6.5	3.4			<0.0001	0.0157	0.0101	10	90	0
6	T9	3.9	2.2	36.1	6.1	44.0	11.9			<0.0001	0.002	<0.0001	20	80	90
7	T10	8.2	3.4	3.7	3.7	3.4	4.1			<0.0001	0.767	<0.0001	30	100	100
8	T11	14.7	12.7	0.8	0.9	5.8	3.9			<0.0001	0.0001	<0.0001	80	100	<5
9	T12	17.2	10.1	9.0	5.4	8.0	4.6			0.0002	0.443	<0.0001	80	NA	100
9	T13	17.2	10.1	9.0	5.2	8.0	4.6			0.0002	0.443	<0.0001	80	NA	100
10	T14	3.2	2.8	0.8	1.6	NA	NA			0.0001	NA	NA	10	100	90
10	T15	3.2	2.8	1.5	1.6	NA	NA			0.0055	NA	NA	50	100	70
10	T16	3.2	2.8	0.0	0.1	NA	NA			<0.0001	NA	NA	50	40	100
11	T17	2.8	2.0	2.6	3.3	28.5	23.0			0.7775	0.0001	<0.0001	70	60	100
12	T18	2.5	1.8	25.6	5.4	20.4	10.1			<0.0001	0.0158	<0.0001	40	25	90
13	T19	24.9	27.9	1.1	1.8	1.2	1.3			<0.0001	0.806	<0.0001	<5	NA	100
14	T20	4.1	1.8	1.6	1.6	5.3	6.8			<0.0001	0.0042	0.354	40	NA	90
15	T21	13.7	7.0	1.2	0.8	NA	NA			<0.0001	NA	NA	45	0	100
15	T22	13.7	7.0	1.4	1.8	NA	NA			<0.0001	NA	NA	75	60	100

NA indicates Not Available.

^{*} The p53 immunostaining results were earlier published.³⁸