Short Communication

Telomere Lengths of Translocation-Associated and Nontranslocation-Associated Sarcomas Differ Dramatically

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Sarcomas can be divided into those with specific translocations displaying monotonous cytomorphology, and those with complex karyotypes and marked cellular pleomorphism. Telomeres contain terminal DNA sequence repeats that maintain chromosomal stability. Telomeres shorten with cell division and may become dysfunctional leading to chromosomal instability. Using a fluorescence *in situ* hybridization/immunofluorescence method to assess telomere lengths in archival tissues we analyzed these two types of sarcomas using paraffin-embedded primary tumor specimens. Tissues from nine sarcomas with characteristic translocations (two synovial sarcomas, two alveolar rhabdomyosarcomas, two desmoplastic round cell tumors, and one each of infantile fibrosarcoma, myxoid liposarcoma, cellular congenital mesoblastic nephroma) and nine without (four malignant fibrous histiocytoomas, two leiomyosarcomas, one pleomorphic rhabdomyosarcoma, one dedifferentiated chondrosarcoma, and one malignant peripheral nerve sheath tumor) were analyzed. In all (nine of nine) cases with specific translocations, which generally have few karyotypic abnormalities, telomere lengths were similar to or reduced compared to surrounding nonneoplastic tissues. In contrast, telomeres in cases lacking specific translocations, which generally contain complex karyotypes, were often found to be dramatically lengthened and heterogeneous. In addition to markedly elongated telomeres, seven of nine (78%) complex cases exhibited large brightly stained regions corresponding to a specific type of promyelocytic leukemia nuclear body found in immortalized cells that maintain telomeres in a telomerase-independent manner [alternative lengthening of telomeres (ALT) pathway]. This phenotype is

unlike that of epithelial neoplasms that typically display complex karyotypes with abnormally short telomeres maintained by the enzyme telomerase. The discovery of heterogeneous telomere lengths and evidence of the ALT pathway in the majority of sarcomas with complex karyotypes supports the existence of a telomere maintenance pathway incapable of full karyotypic stabilization in pleomorphic sarcomas. These findings provide additional molecular-genetic evidence supporting the dichotomous grouping of sarcomas into those with characteristic signature translocations without extensive additional karyotypic abnormalities, and those without such signature translocations that typically display very complex karyotypes, and point to telomere dysfunction as a plausible contributor to the chromosomal aberrations found in complex sarcomas. (*Am J Pathol* 2004, 164:1523–1529)

Telomeres play a key role in the maintenance of chromosomal stability. They are composed of specialized terminal DNA sequence repeats complexed with specific binding proteins and are located at the ends of every normal human chromosome.¹ Telomere shortening is believed to be important in aging, cell senescence, cell replication, and malignant transformation. In contrast, pathological telomere elongation is found in a number of cancer types, either via telomerase enzymatic activity or by an alternative telomerase-independent pathway thought to involve homologous recombination, known as “alternative lengthening of telomeres” or ALT.^{2–4} A defining characteristic of the ALT phenotype is extreme telo-

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mere length heterogeneity with telomeres ranging from very short to very long. Studies of somatic cell hybrids between ALT-positive and telomerase-positive immortalized cells suggest that the two pathways are often mutually exclusive.⁵ When the ALT pathway is functional, nuclei in a subset of cells display aggregates of telomeric DNA that manifest themselves as medium-large brightly stained spots on hybridization with a fluorescently labeled probe specific for the telomeric DNA repeat sequence.⁶ These brightly stained structures have previously been shown to reside in a variant form of the promyelocytic leukemia (PML) nuclear bodies normally present in all cells. In addition to the PML protein and telomeric DNA, these variant PML bodies have also been found to contain telomeric binding proteins (TRF-1, TRF-2), as well as proteins involved in DNA double-strand break repair and recombination (eg, RPA, Mre11/Nbs1/RAD50 complex, RAD51, RAD52). These structures are considered another hallmark of the ALT phenotype, and are referred to as ALT-associated PML bodies (APBs).^{3,4}

As a generalization, sarcomas can be divided into those with simple karyotypes harboring specific translocations and those with complex karyotypes. The former display monotonous cytomorphology and lack anaphase bridges, a mitotic abnormality indicative of previous DNA double-strand breakage, whereas the latter often are pleomorphic and display anaphase bridges.⁷ Unlike epithelial cancers that typically possess abnormally short telomeres stabilized by telomerase, cancers of mesenchymal origin frequently lack telomerase and exhibit hallmarks of the ALT phenotype.^{8–10} Although the ALT pathway provides another mechanism for telomere length maintenance, it also appears to foster chromosomal instability, perhaps because of the presence of very short unstable telomeres on subsets of chromosomes in ALT-positive cells.^{10–12}

The potential clinical consequences of the ALT phenotype remain primarily unknown as few studies to date have addressed this question. In glioblastoma multiforma, the presence of ALT was found to correlate with a more favorable outcome (survival).¹³ However, Ulaner and colleagues⁹ found no difference in survival for osteosarcoma patients when comparing telomerase-positive *versus* ALT cases. Interestingly, those osteosarcoma patients lacking both telomerase and ALT (no telomere maintenance mechanism evident) displayed an increased survival.

A combined fluorescence *in situ* hybridization (FISH)/immunofluorescence method to assess telomere length in paraffin-embedded tissues has been established in our laboratory.¹⁴ This *in situ* method allows for direct telomere length comparison between cells, because telomere length is proportional to fluorescent signal intensity in this assay. In addition, by providing identification of cell types via simultaneous immunohistochemistry, this method has the advantage of eliminating confounding contributions from contaminating normal cells.¹⁵

Because previous studies examining telomere lengths in human sarcomas have relied on pan-genomic Southern blot methodology using tumor homogenates, short-term *in vitro* cell cultures, or established cell lines, we have applied the *in situ* technique to directly assess and

compare primary tumors of the two general types of sarcoma.

Materials and Methods

Paraffin-embedded tissues from nine sarcomas with characteristic translocations (two synovial sarcomas, two alveolar rhabdomyosarcomas, two desmoplastic round cell tumors, and one each of infantile fibrosarcoma, myxoid liposarcoma, cellular congenital mesoblastic nephroma) and nine without (four malignant fibrous histiocytomas, two leiomyosarcomas, one pleomorphic rhabdomyosarcoma, one dedifferentiated chondrosarcoma, and one malignant peripheral nerve sheath tumor) were retrieved from the archives of Johns Hopkins division of surgical pathology. The translocation-associated sarcomas had been previously shown to harbor their respective characteristic translocations, either via karyotyping or positive identification of the relevant fusion gene product.^{16–18} The other lesions were assumed to possess complex karyotypes, as these sarcoma types are known to display chromosome instability/complex karyotypes (Mitelman Database of Chromosome Aberrations in Cancer 2002, <http://cgap.nci.nih.gov/chromosomes/mitelman>).^{19–21}

Telomere-FISH (TEL-FISH) and Telomere/Immunostaining-FISH (TELI-FISH)

The method for *in situ* telomere staining and image analysis has been described in detail previously.¹⁴ Briefly, deparaffinized slides were rehydrated, steam-treated in citrate buffer, and hybridized with a Cy3-labeled telomere-specific peptide nucleic acid (PNA) probe followed by DAPI (4'-6-diamidino-2-phenylindole) counterstaining and imaging, or were processed for indirect immunofluorescence before the DAPI staining using a primary antibody (anti-PML antibody, catalog no. PG-M3; DAKO Corp., Carpinteria, CA) incubated overnight at 4°C followed by application of fluorescent secondary antibody labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR). The PNA probe complementary to the mammalian telomere repeat sequence was obtained from Applied Biosystems (Framingham, MA) and has the sequence (N-terminus to C-terminus) CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. The analogous specificity control PNA, complementary to the *Caenorhabditis elegans* telomere repeat sequence that differs from the human repeat by a single nucleotide, was a generous gift from Dr. Carol Greider (Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD).

Microscopy

Slides were imaged with a Zeiss Axioskop epifluorescence microscope equipped with short arc mercury lamp illumination (Carl Zeiss Inc., Thornwood, NY) and appropriate fluorescence filter sets (Omega Optical, Brattleboro, VT). Internal controls for telomere FISH staining in

sarcomas consisted of endothelial cells, fibroblasts, and lymphocytes within the same tissue sample. Telomere lengths were evaluated by visual assessment of the fluorescent intensities of the telomeric signals, which are proportional to the length of telomeric TTAGGG DNA repeats.¹⁴ Telomeres were directly compared to those of normal-appearing stromal cells within the same tissue section. Each case was scored by each of two authors (EM, AKM) using the following semiquantitative scoring system: lesions with normal telomere intensity had signals comparable to those of normal stromal fibroblasts and endothelial cells. Lesions with (moderately) short telomeres had telomere intensities appreciably dimmer than the normal stroma, but which were still readily detectable. Lesions were considered very short when telomeres demonstrated signals so dim that they were barely perceptible, dimmer than that of virtually any normal stromal cell we encountered in this study. Lesions with long telomeres demonstrated telomere signals that were appreciably brighter than that of stromal cells, and close to that of lymphocytes, which gave consistently bright signals. Lesions in which the signal varied between cells from bright to short, or very short to normal (ie, lesions with telomere signals spanning two scoring categories) were classified as heterogeneous for telomere length.

Information on the presence or absence of anaphase bridges was obtained from previously published work on these sarcoma cases,⁷ as well as additional evaluation of mitotic figures in this study using both DAPI-stained nuclei and hematoxylin and eosin-stained adjacent sections. Criteria for anaphase bridge presence required a well-separated parallel anaphase plate displaying a perpendicularly aligned connecting chromatin filament.

Results

In all (nine of nine) cases with specific translocations, telomere length was similar to or reduced compared to that of surrounding nonneoplastic tissues (Figure 1, A and B). In contrast, malignant cells in seven of nine (78%) cases lacking specific translocations (four malignant fibrous histiocytomas, two leiomyosarcomas, and one pleomorphic rhabdomyosarcoma) displayed dramatically elongated telomeres as well as telomere length heterogeneity, compared to both surrounding normal cells and to tumors with known translocations (Figure 1; C to F), and this difference was statistically significant ($P = 0.009$, Fisher's test). However, in these sarcomas with long telomeres, telomere lengthening was highly variable from cell to cell in many of the cases (Table 1). In addition to markedly elongated telomeres, six of the nine complex cases exhibited brightly-stained intranuclear spots suggestive of APBs.⁶ We performed immunohistochemical testing for co-localized PML protein, a component of APBs. Staining with specific antibodies for PML protein confirmed that these brightly staining foci were indeed APBs in all six cases (six of six, 100%) (Figure 1G). The proportion of cells displaying APB as well as the number and sizes of APB within a given cell were highly variable from case to case (Table 1), ranging from rare detectable

APBs to 40% of cells staining positive. All six cases with APBs exhibited long telomeres. To confirm the specificity of the telomeric fluorescent signals we repeated the staining on directly adjacent tissue sections using a fluorescently labeled PNA probe targeted against the *C. elegans* telomere repeat sequence that differs from the human sequence by a single nucleotide (TTAGGC *versus* TTAGGG). Although *C. elegans* tissue controls exhibited positive staining with the *C. elegans*-specific probe, all human sarcomas were completely nonreactive indicating that the bright signals observed with the human-specific probe in pleomorphic sarcomas, as well as APBs, were indeed reflective of human telomeric DNA (data not shown).

The presence of telomere lengthening and PML bodies also correlated with the presence of anaphase bridges in pleomorphic sarcomas (Figure 2) (Table 1). However, two of the pleomorphic sarcomas lacked sufficient anaphases for evaluation (further details of the anaphase bridge results are published elsewhere⁷).

Discussion

Sarcomas can be divided into two general categories, namely those with specific genetic alterations and those with nonspecific ones.²² Those with specific alterations tend to have simple karyotypes with a specific reciprocal translocation, to occur in younger patients, to lack p53 alterations, and be rare in the Li-Fraumeni, bilateral retinoblastoma syndromes, or the setting after radiation. On routine microscopy, they generally display a homogeneous population of rounded or spindle cells. In contrast, those lacking specific alterations generally have complex karyotypes, no reproducible pattern of translocations, occur in older individuals, have p53 alterations, and are common in patients with Li-Fraumeni and bilateral retinoblastoma syndromes or after radiation. On routine microscopy, their appearances are highly variable but they often display pleomorphic spindle cells.

Pathological telomere lengthening may potentially arise in sarcomas, either because of dysregulated telomerase activity, or by an alternative pathway known as ALT.^{3,4} When the ALT pathway is functional, nuclei of a subset of affected cells display a variant form of PML nuclear bodies, so named based on their disruption in neoplastic promyelocytic leukemia cells. These variant PML bodies, referred to as APBs,⁶ are relatively large structures containing telomeric DNA, telomeric binding proteins (TRF1, TRF2), DNA replication protein (replication factor A), DNA recombination proteins (RAD51, RAD52), and PML protein.

The telomere phenotype of sarcomas with complex karyotypes remains unclear but the ALT mechanism seems to be at work in at least some examples.¹⁰ In this study, the first *in situ* telomere length assessment of these tumor types, we specifically explored the relationship between telomere length, the ALT phenotype, anaphase bridge formation, and sarcoma type. Although several methods for telomere length assessment have been developed, the high-resolution method used in this study¹⁴

Figure 1. TELI-FISH staining of sarcomas using a Cy3-labeled PNA hybridization probe (red). The fluorescent signal intensity is proportional to the amount of telomeric DNA present. In all cases, nuclei are counterstained with DAPI (blue). **A:** H&E; **B:** TELI-FISH. Example of sarcoma with simple karyotype (infantile fibrosarcoma) displaying telomere lengths in the normal range. Note that there is similar telomeric signal intensity between endothelial (arrow) and neoplastic (arrowhead) cells. A cleanly separated anaphase figure is also seen here. **C to F:** Pleomorphic sarcomas displaying long and heterogeneous telomeres and APBs (arrows). **Insets** show H&E-stained malignant fibrous histiocytomas (**C, E, F**) and leiomyosarcoma (**D**). **G:** Co-localization of PML protein (green) and telomeric DNA signals (red) confirms identity of APBs.

Table 1. Summary of Results of Telomere Assessment

Case	Sarcoma type	Grade	Translocation-associated?	Gene fusion product identified?	Status, interval	Telomere lengths	ALT-associated PML bodies (% cells positive)	Anaphase bridges found?
1	Malignant fibrous histiocytoma	3	No	NA	ANED, 1 year	Short-long, heterogeneous	30%	Yes
2	Malignant fibrous histiocytoma	3	No	NA	ANED, 1 year	Short-long, heterogeneous	40%	Yes
3	Leiomyosarcoma	3	No	NA	Alive with lung metastases, 3 years	Short to long, heterogeneous	10%	Yes
3a	Leiomyosarcoma-Separate area from same tumor as case 3	3	No	NA	Alive with lung metastases, 3 years	Long to very long, moderate heterogeneity	25%	Yes
4	Leiomyosarcoma	3	No	NA	Alive with lung metastases, 2 years	Long	≤1%	Not done
5	Malignant fibrous histiocytoma	3	No	NA	Alive with lung metastases, 5 years	Long, Moderate heterogeneity	≤1%	Yes
6	Malignant fibrous histiocytoma	2	No	NA	ANED, 2 years	Long	≤1%	Not done
7	Malignant peripheral nerve sheath tumor	2	No	NA	Dead of metastatic disease, 3 years	Normal long, heterogeneous	No	Yes
8	Pleomorphic rhabdomyosarcoma	3	No	NA	Dead of metastatic disease, 2 years	Very short to short	No	Yes
9	Dedifferentiated chondrosarcoma	3	No	NA	Alive with metastatic disease, 1 year	Short-normal	No	Not done
10	Alveolar rhabdomyosarcoma	3	Yes	Yes, PAX3/FKHR	ANED, 7 years after 3 recurrences	Short-normal	No	No
11	Alveolar rhabdomyosarcoma	3	Yes	Yes, PAX3/FKHR	Dead of metastatic disease, 3 years	Short-normal	No	No
12	Desmoplastic small rounds cell tumor	3	Yes	Yes, EWS/WT1	NA	Short-normal	No	No
13	Desmoplastic small round cell tumor	3	Yes	Yes, EWS/WT1	NA	Very short-normal	No	No
14	Infantile fibrosarcoma	1	Yes	Yes, ETV6/NTRK 3	NA	Normal	No	No
15	Myxoid liposarcoma	1	Yes	Yes, t(12; 16)	Dead of metastatic disease, 4 years	Normal	No	No
16	Cellular congenital mesoblastic nephroma	1	Yes	Yes, ETV6/NTRK 3	ANED, 5 years	Short-normal	No	No
17	Synovial sarcoma	2	Yes	Yes, SYT/SSX	NA	Short-normal	No	No
18	Synovial sarcoma	3	Yes	Yes, SYT/SSX	Dead of metastatic disease, 3 years	Short-normal	No	No

Abbreviations: ANED, alive, no evidence of disease; NA, not available.

is applicable to standard formalin-fixed human tissue, and because the method is *in situ*, lengths in sarcomas can be directly compared to internal controls.

Using TELL-FISH, we find that dramatic telomere lengthening corresponds with pleomorphic sarcomas that typically display complex karyotypes. In contrast, relatively normal, and/or shorter than normal, telomere lengths were observed in neoplasms with characteristic translocations. Furthermore, the proportion of cells displaying lengthened telomeres and ALT was highly variable in the complex cases. For example, in one portion of tumor 3, a leiomyosarcoma, telomeres were within the short-normal range, and APBs were common, whereas a separate focus of this tumor displayed widespread telomere elongation and fewer APBs. Regional variation in

telomere length has been noted previously for liposarcomas by Schneider-Stock and colleagues.²³ Sarcomas with lengthened telomeres and APBs also displayed anaphase bridges. The details of this assessment are reported elsewhere,⁷ but anaphase bridges are known to occur when dicentric chromosomes are produced by chromosomal fusions after DNA double-strand breakage and are considered to be indicators of chromosomal instability. By routine light microscopy of hematoxylin and eosin-stained slides, they are seen as a perpendicularly aligned amphophilic filament connecting a well-separated parallel anaphase plate.⁷ Previously it has been shown that telomere lengths within ALT-positive cells are typically quite heterogeneous, ranging from abnormally long to very short. Because critically short telomeres

Figure 2. Four representative fields of DAPI-stained nuclei showing anaphase bridges (case 5).

become dysfunctional and lead to chromosome fusions, this seems a likely source of the anaphase bridges observed in these ALT-positive cases.²⁴

In the current study we find a correlation between complex karyotypes, abnormally long telomeres, APBs, and anaphase bridges. Of six complex cases in which both APB and anaphase bridges were evaluated, all showed the simultaneous presence of both features. APBs are considered a hallmark of the ALT telomere maintenance phenotype that is typically characterized by extreme telomere length deregulation resulting in widespread length heterogeneity. In sharp contrast, none of the 10 cases with simple karyotypes exhibited APB, anaphase bridges, abnormally elongated telomeres, or significant telomere length heterogeneity as assessed by *in situ* FISH analysis. Little is currently known regarding the potential prognostic significance of telomere maintenance status (eg, presence *versus* absence, type of maintenance mechanism) in sarcomas.^{9,13} In the current study we observed a tendency for more favorable outcomes in those sarcoma cases with evidence of the ALT mechanism among this set of predominantly high-grade sarcoma types. However, one caveat with respect to these findings is that the absolute number of cases examined in this study was small. More extensive research is clearly warranted.

These findings are in contrast to those of epithelial neoplasms in which telomere shortening often accompanies neoplasia²⁵ and appears very early in the disease process.^{26,27} Interestingly, despite the fact that carcinomas typically exhibit telomerase activity (85 to 90%), most fail to display abnormally lengthened telomeres.^{28–30} Because telomerase production by pleomor-

phic sarcomas is variable,³¹ consistent lengthening of telomeres would not be expected in the absence of another mechanism for telomere lengthening and further supports an alternate pathway of telomere lengthening in pleomorphic sarcomas. Such a mechanism has been reported in osteosarcomas and correlates with chromosomal instability and nuclear shape abnormalities.^{10,11,32} Because other lines of evidence show that pleomorphic sarcomas in general display chromosomal instability (in contrast to translocation-associated ones), we suspect that alternative lengthening of telomeres² plays a role in a host of pleomorphic sarcomas. The presence of ALT in pleomorphic sarcomas, plus the observed presence of elongated telomeres, may make them recalcitrant to emergent therapies directed against telomerase. *In situ* telomere length analysis allows for the rapid assessment of telomere lengths, as well as the presence of ALT, in human primary tumor samples and thus should facilitate further examination of the potential prognostic value of these characteristics in human sarcomas.

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