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Telomerase Activity in Human Cancer
Final Report

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The overall goal of this collaborative project was to investigate the role in malignant cells of both chromosome telomeres, and telomerase, the enzyme that replicates telomeres. Telomeres are highly conserved nucleoprotein complexes located at the ends of eucaryotic chromosomes. Telomere length in somatic cells is reduced by 40-50 nucleotide pairs with every cell division due to incomplete replication of terminal DNA sequences and the absence of telomerase, the ribonucleoprotein that adds telomere DNA to chromosome ends. Although telomerase is active in cells with extended proliferative capacities, including more than 85% of tumors, work performed under this contract demonstrated that the telomeres of human cancer cells are shorter than those of paired normal cells, and that the length of the telomeres is characteristic of particular types of cancers. The extent of telomere shortening ostensibly is related to the number of cell divisions the tumor has undergone. It is believed that ongoing cell proliferation leads to the accumulation and fixation of new mutations in tumor cell lineages. Therefore, it is not unreasonable to assume that the degree of phenotypic variability is related to the proliferative history of the tumor, and therefore to telomere length, implying a correlation with prognosis. In some human tumors, short telomeres are also correlated with genomic instabilities, including interstitial chromosome translocation, loss of heterozygosity, and aneuploidy. Moreover, unprotected chromosome ends are highly recombinogenic and telomere shortening in cultured human cells correlates with the formation of dicentric chromosomes, suggesting that critically short telomeres not only identify, but also predispose, cells to genomic instability, again implying a correlation with prognosis. Therefore, telomere length or content could be an important predictor of metastatic potential or responsiveness to various therapeutic modalities.

The standard method for the measurement of the terminal restriction fragment (TRF) is Southern blot analysis using a telomere-specific DNA probe. However, there are three significant limitations to this method. First, Southern blot analysis of telomere DNA typically requires 1-10 ug of DNA per sample. Second, DNA breakage reduces the observed telomere length. Finally, the TRF includes telomere-associated DNA other than the TTAGGG sequence. To circumvent these problems, we developed an alternative assay for telomere DNA content, a proxy for telomere length, in which the DNA is analyzed by slot blotting (Bryant et al, Biotechniques 23: 476, 1997). In this approach, duplicate blots are probed with oligonucleotides specific for telomere and centromere DNA sequences. The hybridization intensity for each sample is quantitated with a phosphorimager. The intensity of the telomere signal is dependent on the amount of DNA in the sample and the length of the telomeres. The intensity of the centromere signal is dependent on only the amount of DNA in the sample. Thus, the data is expressed as the ratio of the telomere:centromere (T:C) intensities to normalize for differences in the absolute amounts of DNA between samples. In control experiments involving HeLa cell (cancer cell) and placental (normal cell) DNA, the T:C ratio in the HeLa DNA samples was 57% of the ratio in the placental DNA. By comparison, the mean telomere lengths measured by Southern blotting in the HeLa cell DNA were 55-60% of the telomere length in placental DNA.

To further validate this measurement of telomere DNA content, T/C ratios were measured in 30 normal and cancerous human DNA samples with known differences in modal TRF length. The relative content of telomere DNA was directly proportional to telomere length ($r = 0.904$). At a T/C ratio of zero, the TRF length was estimated to be 0.7 Kbp. Consistent with this result, non-TTAGGG telomere-associated DNA sequences, strings of distinct repeated sequence elements collectively comprising 0.5-1.0 Kbp, are associated with approximately 22 human telomeres . The TTAGGG sequence also exists outside of the

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telomere. However, because the telomere DNA content and telomere-associated DNA together account for the observed TRF length, it is unlikely that interstitial TTAGGG sequences significantly affect the background of the assay.

Although several reports have correlated telomere length with genetic variability, progression and aggressiveness, the value of telomere length as a prognostic tool has not been proven. A definitive answer to this question could be obtained by retrospective measurements of telomere length in archival paraffin-embedded biopsy or tumor specimens from patients where the course of disease and outcome are known. In this connection, the UNM Cancer Research and Treatment Center (CRTC) Solid Tumor Facility and Center for Molecular and Cellular Diagnostics possess extensive repositories of well-characterized leukemias, lymphomas and solid tumors. In addition, the CRTC houses the New Mexico Tumor Registry (NMTR), one of nine National Cancer Institute Surveillance, Epidemiology and End Result (SEER) programs in the country since 1973. The NMTR database contains about 120,000 confidential cancer patient records and it increases by more than 6,000 newly developed cases each year. Vital status of cancer cases is monitored to maintain survival data. The NMTR links its database of anonymous patient histories to the locations of frozen and formalin-fixed, paraffin embedded tumor specimens through hospital pathology reference numbers and dates of procedures, providing a unique resource for retrospective investigations of the prognostic value of telomere content.

Southern blot analysis of telomere length requires approximately 5 mg of undegraded DNA. Because there is only a small amount of tumor tissue in the paraffin block, and the DNA isolated from paraffin blocks is frequently degraded, the DNA purified from archival paraffin sections is not suitable for Southern blot analysis of telomere length. In contrast, our studies have demonstrated that quantification of the content of telomere DNA by slot blot is independent of DNA breakage, DNA ploidy, DNA sample size and can be performed on as little as 10-20 ng of total DNA, making it particularly well suited for such retrospective investigations (Bryant et al, Biotechniques 23: 476, 1997).

We used the slot blot assay to investigate the relationship between telomere length, genetic instability and prognosis. A preliminary study was a comparison of telomere content in 50 frozen samples of aneuploid and diploid breast carcinomas. The results indicated that reduced telomere content is strongly correlated with aneuploidy ($p < 0.005$) consistent with the interpretation that short telomeres predispose to genetic instability (Griffith et al, submitted). Importantly, subsequent chart review also indicated that the metastatic potential of the tumors, as defined by lymph node involvement was correlated with reduced telomere content, as was the incidence of tumor recurrence. In other words, tumors with the least telomere DNA appeared the most likely to metastasize and recur.

A preliminary retrospective investigation of the prognostic significance of telomere content in prostate cancer was also funded by this contract. The NMTR identified two groups of ten patients that had identical prognostic markers at the time of their prostatectomies (age of patient, tumor size and grade, Gleason score and prostate-specific antigen (PSA) levels.), but different outcomes (alive or deceased after five years). The ten patients that did not survive five years had the ten lowest contents of telomere DNA while the patients that did survive had the ten greatest contents of telomere DNA. The mean telomere content in the surviving group was more than twice that in the deceased group and overlapped the content of telomere DNA in the adjacent normal tissue from the same patient (Donaldson et al, in preparation). contrast, there was no correlation between telomere content in prostate cancer and loss of heterozygosity (Bova et al, in preparation). This is consistent with the view that telomere shortening predisposes to chromosome breakage and fusion, and not mitotic recombination.

One mechanism to explain the deleterious consequences of telomere shortening in prostate cancer is the inactivation of terminal gene sequences that are required for normal growth control. Using the highly sensitive slot blot assay, we recently began analyzing telomere content in flow-sorted chromosomes from prostate cell lines in collaboration with Dr. Scott Cram at LANL and Dr. Steven Bova at the Johns Hopkins University School of Medicine. The initial results indicate that chromosomes 13 and 19 have different T/C ratios, suggesting the intriguing possibility that either the rate of telomere shortening or the steady state content of telomere DNA is not uniform throughout the genome.

Telomerase, the enzyme that adds telomeres to chromosomes, may also play an important role in malignant transformation. Normal cells have a limited proliferative capacity and do not contain telomerase activity. In contrast, tumor cells have an unlimited capacity for cell division. To prevent excessive telomere shortening, it has been hypothesized that it is necessary for tumor cells to reactivate telomerase, which is normally expressed only during early embryonic development and in stem cells. Consistent with this hypothesis, more than 85% of tumors contain a telomerase-like activity. The seemingly essential role of telomerase in maintaining chromosome integrity and its nearly ubiquitous occurrence in human cancers have made telomerase a potential target for anti-cancer therapy.

We constructed two retroviral vectors designed to express a stable mRNA molecule containing either "sense" or "antisense" telomere sequence. Asynthetic oligonucleotide containing a translation terminator signal (TGA) followed by six copies of either a TTAGGG (sense) or CCCTAA (antisense) telomere sequence was inserted into the IL-7 gene of a recombinant IRES/HyTK retrovirus. This vector exploits an internal ribosome entry sequence (IRES) so that two proteins are translated from a single mRNA species. Because the coding sequence for hygromycin phosphotransferase is colinear and 3' to the telomere oligonucleotide, selection for cells containing retroviruses which express resistance to the antibiotic hygromycin coselects for cells expressing the upstream telomere sequence.

The recombinant retroviruses were transfected into the murine packaging cell line PA317 using the calcium phosphate method. DNA was purified from hygromycin resistant clones and the presence of the correct recombinant retrovirus was confirmed by PCR analysis. Viral supernatants were produced from several clones of the PA317 cells and used to infect transduced Hela cells, A498 human renal carcinoma cells and human small cell lung carcinoma cells, all of which produce telomerase and have shortened telomeres, and early passage human fibroblasts, which do not produce telomerase and do not have shortened telomeres.

The retroviruses expressing the antisense telomerase RNA sequence significantly inhibited telomerase activity in all cell lines, in some instances to undetectable levels. In contrast, there was no difference in the levels of telomerase activity in cells transfected with the sense-containing retrovirus. Surprisingly, telomere DNA content and cellular proliferation and viability were not affected by the absence of telomerase activity for 50 population doublings, indicating the existence of telomerase-independent mechanism of telomere maintenance (Bisoffi et al, in press).