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Special Session 1

Telomeres and telomerases

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General concepts and technical considerations

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Telomeres are a noncoding repetition of hexameric sequences (TTAGGG)n, located at the end of the arm of each chromosome. They protect chromosome ends against fusion, recombination and degradation. After each cell division, telomeres shorten because the DNA polymerase cannot replicate the very end of the linear DNA strands (the "end replication problem"). *In vitro,* when telomere length shortens and reaches a critical size, the cell ceases to divide and enters senescence. However, under oncogenic stimulation, a few cells can overpass senescence and finally stabilize telomere length, allowing cells to divide indefinitely. This event parallels the activation of a ribonucleoproteic complex, telomerase, an enzyme able to add de *novo* telomeric hexamers at the end of DNA strands. This reverse transcriptase is constituted mainly by an RNA component (hTR) that serves as a template, and by a proteic catalytic subunit (hTERT). Telomerase is detected in embryonic cells and stem cells in adults but is absent in most normal somatic cells. It has been proposed that the activation of telomerase, through its effect on telomere elongation, may be required to sustain the indefinite proliferation of malignant cells. Indeed, using the telomeric repeat amplification protocol assay (TRAP), a highly sensitive method allowing the detection of telomerase activity from tissue extracts, many studies have shown the presence of telomerase in the majority of human malignant tumors.

Although the TRAP assay is very sensitive, it is performed on tissue extracts containing a heterogeneous population of cells, including tumoral but also inflammatory cells, such as lymphocytes. These cells are known to exhibit telomerase activity when activated. Several attempts have been made to develop *in* situ techniques allowing the evaluation of topographic and cellular distribution of telomerase activity as well as telomere length. These techniques should also allow the analysis of telomerase expression or telomere length in the context of morphological modifications. Using a FISH protocol with telomeric probes in combination with an automatic measurement of fluorescence intensity, it is now possible to assess telomere length on cytologic or histologic preparations. Attempts to assess telomerase expression indirectly have been performed by *in situ* hybridization of hTR and hTERT messenger RNA (mRNA), the two major components of telomerase. However, neither hTR nor hTERT mRNA expression correlates strictly with telomerase activity. More recently, the development of hTERT antibody and an *in situ* protocol derived from the TRAP assay have generated some interest in the study of the cellular site of telomerase expression during liver carcinogenesis in humans.

The telomere/telomerase hypothesis is a major new concept in biology but the question of whether telomerase expression is the prime mover or only a bystander in carcinogenesis has not yet been settled.

As for other cancers, telomerase activity has been detected in hepatic cell carcinoma (HCC) by TRAP assay in 80-91% of cases. Telomerase activity is also present in more than 80% of nodular precancerous hepatic lesions (macronodules), whereas it is rarely detected in cirrhotic tissues. Although the TRAP assay is very sensitive, it is performed on tissue extracts containing a heterogeneous population of cells, including tumoral but also inflammatory cells, such as lymphocytes. These cells are known to exhibit telomerase activity when activated, a pitfall particularly relevant for the evaluation of telomerase in chronic hepatitis and active cirrhosis.

There is therefore a need for an *in situ* technique, which would allow the evaluation of topographic and cellular distribution of telomerase expression. This technique should also allow the analysis of telomerase expression in the context of the morphological modifications observed during carcinogenesis. Attempts to assess telomerase expression indirectly have been performed by *in situ* hybridization of hTR and hTERT mRNA, the two major components of telomerase. However, neither hTR nor mRNA expression correlates closely with telomerase activity. hTR is widely expressed even in the absence of telomerase activity. hTERT mRNA detection might be more closely related to telomerase but some discrepancies also exist because of alternative splicing, post-transcriptional modifications and enzyme activation. In the present study, we developed an *in situ* protocol derived from the TRAP assay allowing the direct visualization of telomerase activity on frozen tissue section. The *in situ* TRAP assay was used to study the cellular site of telomerase expression during liver carcinogenesis in humans.

Telomerase activation in human tumors

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It has been known for some time that cells that replicate lose some of their telomeric DNA. A popular hypothesis is that cellular and germ cell senescence is a result of this telomeric 'erosion". Stem cells have an enzyme system, telomerase, that restores the lost DNA, which consists of repeats of the sequence TTAGGG. Telomerase is a reverse transcriptase, consisting of an RNA template (TR) and a catalystic protein (TERT). The discovery that telomerase, which purportedly contributes to the unlimited life span of the cancer cell, is activated in many cancers and in almost all cancer cell lines has

raised great interest because of its diagnostic (but also therapeutic) potential.

In order to be of diagnostic use, the telomerase system must be accessible for efficient and relatively simple routine analysis in biopsy and surgical specimens. The first test that became available was the telomeric repeat amplification protocol (TRAP), which uses telomerase's ability in a sample to synthesize telomeric repeat fragments on a provided template. The repeats obtained are amplified by polymerase chain reaction (POR) and separated by electrophoresis, which results in a ladder pattern. For surgical specimens, quality control of the tissue is essential. Warm ischemia during operation or the time lapse between the operation and the sampling of a specimen can lead to RNA degradation, which may yield a potentially false negative TRAP result. We routinely use rRNA as a simple test for RNA integrity. The 18 and 28 SRNA bands should be visible for a sample to be accepted in the TRAP assay. More recently, a variety of tests for the presence of hTR and hTERT have been developed. Both can be detected by *in situ* hybridization in routinely processed tissue specimens but also by reverse transcription (RT)-PCR. For the RT-PCR of hTERT a significant problem is the fact that the gene consists of a single exon. The presence of genomic DNA is therefore a potential confounding factor. We routinely predigest the sample with deoxyribonuclease to circumvent this problem.

With these tools we have addressed three relevant problems in diagnostic pathology: i) the elucidation of the adenoma-carcinoma sequence in the colorectum; ii) the classification of soft tissue tumors; and iii) the detection of malignant cells in effusion fluids.

In colorectal tissues we studied telomerase activation by the TRAP assay. This study included carcinomas, adenomas and normal mucosa. Almost 30% of the tissues were not suitable for analysis because of RNA degradation. In the remaining samples, telomerase was not found in normal mucosa. Of the adenomas, 47% were telomerase-positive as were 100% of the tested carcinomas. In the adenomas, we compared telomerase activity with the degree of dysplasia, as assessed in a section consecutive to that used for the TRAP assay. With this approach, a high degree of dysplasia correlated strongly (<0.0001) with telomerase activation. We also compared telomerase activation with *K-ras* and p53 mutations. The frequency of telomerase activation was higher than that of K-ras mutation but lower than that of p53 mutation, which suggests that in the molecular carcinogenesis sequence telomerase activation occurs later than *K-ras* mutation but earlier than p53 mutation.

In soft tissue tumors, we analyzed telomerase activity by TRAP as well as hTERT by RT-PCR in benign lesions, low-grade sarcomas and high-grade sarcomas. Telomerase was undetectable in the benign lesions and low-grade sarcomas but was activated in 50% of the high-grade sarcomas. This indicates that, although telomerase activity is synonymous with malignancy in soft tissue tumors, it does not help in making a distinction between reactive or benign lesions and low-grade lesions. Some high-grade tumors, most notably leiomyosarcomas and malignant fibrous histiocytomas, never showed telomerase activity. This was not due to the presence of telomerase inhibitors, nor was it related to proliferative activity. We observed a strong correlation between telomerase activation and hTERT messenger RNA expression.

We also studied telomerase activity in a series of pleural, pentoneal and pericardial effusions, in parallel with cytology. Of these samples, 30% were cytologically malignant and 70% also had telomerase activity; consequently 30% of the cytologically malignant cases had no telomerase activity. However, in most of these cases the RNA was almost completely degraded. About 20% of the cases had negative cytology but telomerase activity. Of these, more than half concerned patients with disseminated cancer, which might suggest that telomerase activity could be a more sensitive parameter for malignant cells in an effusion than in cytology. However, several cases were telomerase-positive without any clinical indication of cancer. Only time will tell whether or not these are to be regarded as lalse positive". These data indicate that telomerase activity might become a useful parameter in the analysis of cytological specimens.

Altogether, telomerase analysis appears to hold great promise in the analysis of (pre)neoplasia in cytological, biopsy and surgical specimens. Larger prospective studies are needed to determine the impact of telomerase analysis on cancer diagnosis.