RECTIFICATIE

TELOMERASE IN HUMAN CANCER

TELOMERASE IN TUMOREN BIJ DE MENS

Proefschrift

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van het Institut Universitaire de Pathologie, Faculté de Médecine,
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onder leiding van dr.Jean Benhattar

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CONTENTS

LIST OF ABBREVIATIONS

ALT	alternative mechanisms for lengthning of telomeres
APB	ALT-associated PML body
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
HG	high grade dysplasia
LG	low grade dysplasia
PML	promyelocytic leukemia
RT-PCR	reverse transcriptase polymerase chain reaction
SSCP	single strand conformation polymorphism
STS	soft tissue sarcoma
TA	telomerase activity
TP1	human telomerase associated protein
TRAP	telomeric repeat amplification protocol

CHAPTER I

General introduction and outline of the study

1.1 General introduction of telomerase in human tumours

1.1.1 Telomerase and telomere

1.1.1.1 The functions of telomeres

The chromosome ends are specialized nucleoprotein structures called telomeres which "cap" the termini of linear eukaryotic chromosomes and stabilize them. In humans, telomeres are made up of an average of 5000-15,000 base pairs of $\{TTAGGG\}_n$ repeats and telomere-binding proteins. Telomeres form specific complexes with telomere-binding proteins. They are involved in chromosome replication, nuclear architecture, chromosome stability, gene expression, human tumour formation, aging and cell division (Greider, 1996; Blackburn, 1997). Owing to an inherent flaw in the way cells copy their DNA, each time a cell divides it loses 50-100 base pairs at the end of its telomeres. This is known as the end-replication problem (Figure 1), as first described by Watson (Watson, 1972).

The end replication problem predicts the progressive loss of chromosomal DNA at the 3' ends over multiple cycles of replication. When a telomere loses a critical number of base pairs, it triggers a signal for the cell to stop dividing and senesce (Harley et al, 1990). Thus, telomere reduction is thought to be a biologic clock regulating the life span of a cell. Many unicellular organisms and viruses have evolved a special mechanism to circumvent the problem of termini. In these organisms, the chromosomes are circular, or the genome produces circular replicative intermediates that simply lack ends so that the problem encountered in linear chromosomes does not exist (Hayflick, 1997). But the most common solution in higher eukaryotes is an enzyme complex called telomerase, a specialised reverse transcriptase that synthesises telomeric DNA sequences and thus restores telomere length (Morin, 1989; Nakamura et al, 1997). This process maintains a dynamic equilibrium and prevents the chromosomes from shortening to a critical length and prevents cells from receiving the signal to stop dividing.

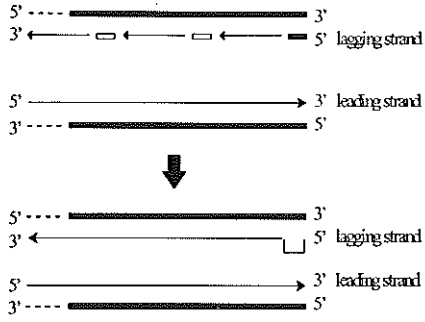

Figure 1. End replication problem. Original chromosome strands are depicted in black thick lines. As the replication fork proceeds from left to right, the leading strands (rightward arrow) proceeds continuously to replicate one strand of original DNA. The direction of the lagging strand is opposite to the direction of the replication fork and relies on the ligation of Okazaki fragments (leftward arrows), which are primed with short stretches of RNA (empty boxes). Most RNA primers are replaced with DNA from an upstream Okazaki fragment, but the terminal RNA primer (solid box) is never replaced with DNA. Consequently, each round of replication produces daugher chromosomes, which lack the sequences corresponding to the original 3' ends (bracket).The terminal primer actually may not anneal to the extreme 3' end, contributing to further loss of end sequences. (Figure cited from Rhyu, 1995).

1.1.1.2 The telomerase-telomere complex

In human, telomerase comprises of several protein subunits and an RNA component. The RNA component (human telomerase RNA, **hTR**), contains a domain that is complementary to one hexameric unit of the DNA telomeric repeat sequence, TTAGGG (Feng et al, 1995). The protein moiety contains the catalytic subunit (human telomerase reverse transcriptase, **hTERT**) that is homologous to reverse transcriptases (Nakamura et al, 1997). Another moiety of protein (human telomerase associated protein, **TP1**), was the first identified protein component, but the function as yet remains unknown (Harrington et al, 1997). Thus, the enzyme complex comprises both template and polymerase activity. A simplified model of the telomerase-telomere reaction is shown in Figure 2.

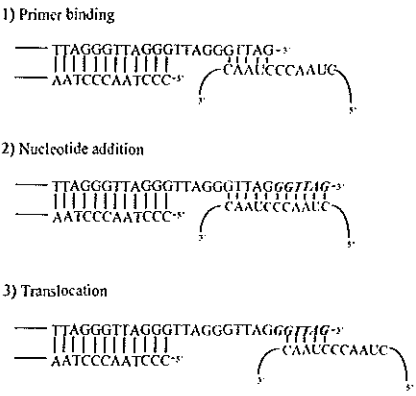

Figure 2. Mechanism of telomere addition. Step 1: 3' end of chromosomal DNA binds to the part of template region in telomerase RNA. Step 2: Telomerase RNA provides the template for elongating the 3' end of the chromosome (newly synthesized sequence in black and italics). Step 3: The chromosome is translocated and repositioned to repeat the polymerization step.

1.1.1.3 Human telomerase, senescence and immortality

Olovnikov was the first to suggest that gradual loss of chromosome ends could lead to an exit from the cell cycling compartment (Olovnikov, 1973). Subsequently, Harley and coworkers (Harley et al, 1990; 1992) documented the relation between replicative senescence, immortalization and the shortening of telomeres. In human cells, cellular senescence can be divided into two parts: Mortality stage 1 (M1) and Mortality stage 2 (M2). Immortalization of cells is associated with activation of telomerase at or near crisis in the M2 stage, as shown in Figure 3. In view of the substantial variation of terminal restriction fragments (TRF) within a cell population, it has been proposed that senescence occurs when one or more TRF reaches a critical length (Allsopp and Harley, 1995). Inactivation of p53 and RB genes allows cells to continue to proliferate, despite critically short TRFs (Hara et al, 1991; Wazer et al, 1995). But it is not known how these tumour suppressor proteins act in this capacity. They are proposed to prevent cells from sensing, or allow cells to bypass, the signal(s) generated by a critically short telomere. Also little is known as to how critically short telomeres result in cellular senescence. Possibly, cells respond to a short telomere as they do to DNA damage (Goldstein, 1990; Harley and Villeponteau, 1995).

Alternatively, a critically short telomere may sequester transcription factors that can activate or reactivate a variety of genes (Marcand et al, 1996).

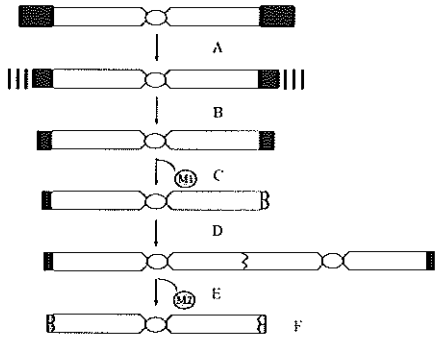

Figure 3. Model for telomere role in senescence and immortality. (A) In somatic cells, telomerase activity is turned off by unknown mechanism(s). During cell divisions, telomeres continue to shorten. (B) Telomere shortening reaches a critical point. (C) Some cellular factor(s) detect critically short telomeres, resulting in M1 crisis or exit from cell cycle and cells senesce. (D) Mutations in genes detecting genetic damage or regulating the cell cycle allow a clonal cell population to continue to divide and escape senescence, resulting in further telomere loss and chromosomal instability. (E) Further cell division leads to M2 crisis and most cells die. (F) Rare cells require telomerase activity and their telomeres are stabilized. These cells are immortal. (Figure cited from Rhyu, 1995)

1.1.1.4 TRAP assay

A critical step in the evolution of telomerase studies has been the development of a specific and sensitive method for the detection of telomerase activity. The general used Telomeric Repeat Amplification Protocol (TRAP) is a very sensitive assay (Kim et al, 1994). It can detect telomerase activity in as few as 1-10 positive cells and 0.01% positive cells in a mixed population, thereby allowing the use of very small amounts of tissue (Wright et al, 1995). However, as pointed out by Meeker and Coffey (Meeker and Coffey, 1997), there are many pitfalls in assaying telomerase activity, including inadequate handing of samples, sampling artefacts, contamination with telomerase positive material and PCR inhibitors. Imperfectly preserved tissue samples and *Taq* polymerase inhibitors are considered as the two main reasons for false-negative results in telomerase activity assays. An internal control is included in the

conventional TRAP assay, to test for *Taq* polymerase inhibitors, whereas little attention has been paid to potential false-negative results due to inadequately preserved tissue samples. As telomerase, a ribonucleoprotein enzyme, uses its RNA as a template and its catalytic subunit as reverse transcriptase for the synthesis of the TTAGGG repeats, it stands to reason that the assay functions only when the protein is active and the RNA is non-degraded. These two conditions indicate that the quality of the tissue is of vital importance for the reliability of the telomerase activity assay.

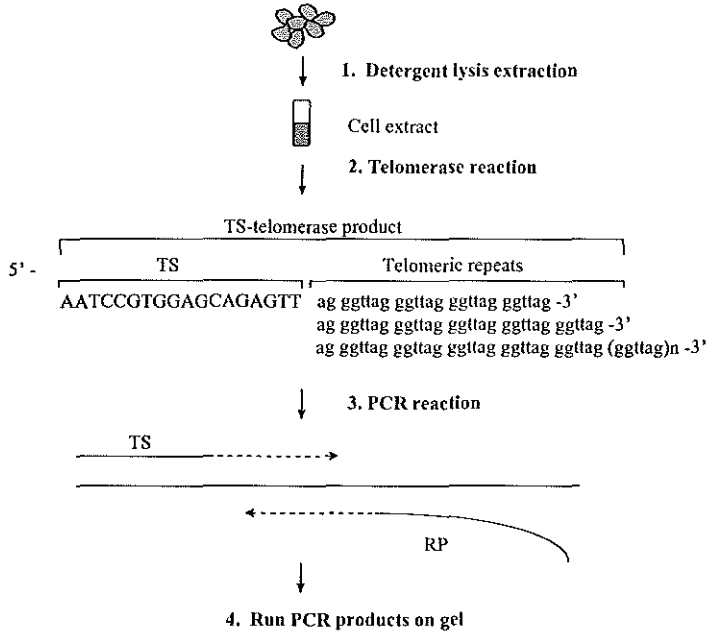

Figure 3. TRAP assay for telomerase activity. Step 1. An extract is prepared from fresh tissues or cells by detergent lysis buffer. Step 2. Addition of telomeric repeats by telomerase. Telomerase adds a number of telomeric repeats (GGTTAG) on to the 3' end of a substrate oligonuleotide (TS) using added deoxynucleotide triphosphates (dNTPs). Since telomerase is a processive enzyme, the products of this reaction will be heterogeneous in length. Step 3. Amplification of TS-telomerase product by PCR. The extended products are amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base increments. Step 4. The amplified products are run on a polyacrylamide gel.

1.1.2 Telomerase components

As mentioned above, telomerase consists of at least three components: hTR, hTERT and TP1. Their functions and the regulatory mechanisms by which telomerase is activated have not been fully determined. The detection of individual telomerase components requires methods to assay hTR RNA, hTERT mRNA and hTERT protein. Molecular biological and histochemical methods for their detection have meanwhile been developed.

1.1.2.1 Telomerase associated protein (TP1)

The presence of TP1, of which the function as yet remains unknown, is not correlated with telomerase activation. It is ubiquitously expressed (Ramakrishnan et al, 1998; Takakura et al, 1998). Thus, TP1 apparently does not play a crucial role in telomerase activation. It could be required *in vivo* for full telomerase assembly, function and functional regulation, but its presence can not be used as an indicator of telomerase activation.

1.1.2.2 Telomerase RNA

hTR, the first component identified, acts as a template for telomere elongation by telomerase. In reconstituting human telomerase activity, hTR is an essential component (Weinrich et al, 1997; Beattie et al, 1998). Tumour cells transfected with antisense hTR lose telomeric DNA, resulting in cellular senescence (Feng et al, 1995). These findings suggest that hTR is indispensable for telomerase activity. It is conceivable that telomerase activity correlates with hTR expression. However, controversial results have been reported regarding this issue. A correlation has been found between telomerase activity and hTR expression, as determined by in situ hybridization; but the level of telomerase activity did not parallel the level of hTR expression (Soder et al, 1998; Heine et al, 1998; Paradis et al, 1999; Maitra et al, 1999). Recent studies demonstrated that hTR RNA, detected by RT-PCR, is widely expressed in both cancerous and non-cancerous tissues, although in cancer tissue at a higher level (Ramakrishnan et al, 1998; Takakura et al, 1998; Kyo et al, 1999; Sumida et al, 1999). The fact that the hTR gene has no introns requires specific consideration. hTR cDNA is identical to genomic hTR DNA and, as it is very difficult to avoid the

presence of traces of genomic DNA in extracted RNA, during PCR amplification genomic hTR DNA might be co-amplified, leading to false-positive results. This might explain why, by RT-PCR, hTR RNA has been found in cancer and non-cancer tissues, irrespective of telomerase activity. This problem can be circumvented by extensive DNase digestion of the extracted RNA before cDNA synthesis. Very few RT-PCR experiments were performed with the use of DNase before cDNA synthesis (Müller et al, 1998; Yasui et al, 1998; Dome et al, 1999; Stanta et al, 1999). In a recent report, normal urine samples were analysed and hTR was detected in only 15% of them whereas 83% of the urine samples from the patients with bladder cancer were found hTR-positive (Müller et al, 1998). In gastric tissues, Yasui et al have observed that telomerase activity was associated with increased hTERT and hTR expression (Yasui et al, 1998). Unfortunately, in the other two papers, no normal tissues were analysed (Dome et al, 1999; Stanta et al, 1999).

1.1.2.3 The reverse transcriptase subunit

hTERT has been identified as the putative human telomerase catalytic subunit (Nakamura et al, 1995). The expression of hTERT is closely correlated with telomerase activity *in vitro* and *in vivo* (Nakamura et al, 1995; Meyerson et al, 1997). Transfection of plasmids encoding hTERT resulted in telomerase activity, telomere maintenance, and extension of life span in foreskin fibroblasts and retinal epithelial cells (Bodnar et al, 1998; Morales et al, 1999). More recently, Hahn and coworkers reported that the ectopic expression of hTERT in combination with two oncogenes (the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras) resulted in direct tumorigenic conversion of normal human epithelial cells and fibroblasts (Hahn et al, 1999). Regulation of the hTERT expression has been proposed as the major determinant of the regulation of the enzymatic activity. hTERT mRNA, determined by either RNase protection or RT-PCR analysis, appears to be expressed at high levels in primary tumors, cancer cell lines, and telomerase-positive tissues but absent in telomerase-negative cell lines and in differentiated tissues (Nakamura et al, 1995; Meyerson et al, 1997). hTERT mRNA has also been found in some telomerase-negative normal tissues in other studies (Ramakrishnan et al, 1998; Tahara et al, 1999). By in situ hybridization, hTERT was present not only in carcinoma tissues but also in

normal epidermis, even at high levels in the basal layer of the epidermis (Kolquist et al, 1998 Nakano et al, 1998). By immunohistochemistry, hTERT protein was found in all crypt epithelial cells of normal colon mucosa except those at the top, although at a lower level than in cancer cells. In crypts more cells were hTERT positive than Ki-67 positive (Takara et al, 1999).

1.1.3 Human telomerase and cancer

The telomere-telomerase hypothesis of cell aging has rapidly become a focus of intensive research in the field of carcinogenesis. The hypothesis is based on the observation that telomerase activity is expressed in embryonic cells, in adult germline cells (Kim et al, 1994; Wright et al, 1996), and in most human tumours (Kim et al, 1994; Avilion et al, 1996; Shay et al, 1997), but is undetectable in normal somatic tissues except for proliferative cells of renewable tissues including haemopoietic stem cells (Hiyama K et al,1995; Broccoli et al, 1995; Chiu et al, 1996), activated lymphocytes (Hiyama K et al,1995), basal cells of the epidermis (Taylor et al, 1996; Harle-Bachor et al, 1996) and intestinal crypt cells (Hiyama E et al, 1996). Meeker summarised more than 2600 human tumour samples that have been tested for telomerase activity using TRAP assay. The overall prevalence of 84.9% makes telomerase activity the most common biochemical marker of human cancer (Meeker et al, 1997). However, telomerase activity as a diagnostic parameter is confounded by the presence of the enzyme in certain benign lesions or in normal tissues. For example, telomerase is activated in up to 95% of breast cancers, absent in normal breast (Carey et al, 1998), detectable in 60% of benign fibroadenomas (Poremba et al, 1998). In lymphoma (Yashima et al, 1997), skin cancer (Taylor et al, 1996) and ovarian cancer tissue samples (Murakami et al, 1997), significant numbers of normal cells were found with telomerase activity. Quantitation of telomerase activity might help to resolve this issue.

1.1.3.1 Telomerase activity in colorectal carcinogenesis

Telomerase is activated in some premalignant lesions, such as hyperplasia and dysplasia in the lung (Breslow et al, 1997), dysplasia and carcinoma in situ in the oropharynx lesions (Califano et al, 1996), as well as in premalignant skin lesions

(Parris et al, 1999). However, at which moment in the multistep pathogenesis of cancer telomerase is activated remains to be clarified. If telomerase could be used to detect preinvasive neoplasia, this could then be treated with greater efficiency and lesser toxicity than for invasive cancer.

Colorectal adenoma-carcinoma sequence is one of the most elaborated models for multiple step tumourigenesis. Tissue samples from different stages can be obtained relatively easily, which has allowed characterisation of the histopathological steps and the accompanying genetic alterations. Gradual progression from adenoma to carcinoma has also allowed to identify at which step telomerase is activated. Telomerase activity has been found in almost all colorectal carcinomas, irrespective of tumour stage and histological type. The reported incidence of telomerase activity in colorectal adenomas varied from 0-100% (Chadeneau et al, 1995; Avillion et al, 1996; Tahara et al, 1995; Tang et al, 1998). Telomerase activity was detected in 50% of colorectal precancerous lesions [hyperplastic polyps (15%), villous adenomas (50%) and tubular adenomas (45%)] by Tahara (Tahara et al, 1995). Chadeneau and Avillion, however, reported absence of telomerase activity in colorectal adenomas (Chadeneau et al, 1995; Avillion et al, 1996). Another group found telomerase activity in 16% of small (<1.0cm) polyps, 20% of intermediate polyps and 71% of large (>2cm) polyps (Tang et al, 1998). Fang and coworks detected, in a series of colorectal tissues obtained by biopsy, telomerase activity in 88.5% (46/52) of colorectal carcinomas, in 50% (6/12) of colonic adenomas but not in normal colorectal mucosa (Fang et al, 1999). No correlation was observed between telomerase activity and tumour location, type, size or differentiation. Telomerase activity has not been studied in detail in normal colon mucosa, but has been found in the bowel wall. It has been suggested that the enzyme activity might originate from stem cells in the lower third of the mucosal crypts or from lymphotytes in the submucosa (Hiyama E et al, 1996; Breslow et al, 1997). Telomerase activity occurs in adenomas but no data shows unambiguous correlation between telomerase activity and grade of dysplasia in colorectal adenomas.

1.1.3.2 Telomerase activity in noninvasive or minimally invasive samples

Telomerase activity has been detected in almost any type of clinical specimen, including exfoliated cells and fine needle aspirates. For example, it has been detected

in oropharynx in the context of head and neck cancer (Califano et al, 1996); in colonic washings in search of coloretal cancer (Yoshida et al, 1997 A); cervical scrapings in search of cervical cancer (Kyo et al, 1997) and in bladder washings or urinary samples for urinary tract cancer (Kavaler et al, 1998; Yoshida et al, 1997 B); and also in fine-needle aspiration biopsies in search of breast, prostate, thyroid and liver cancers (Poremba et al, 1998; Lin et al, 1998; Haugen et al, 1997; Nakashio et al, 1997). One of the limitations of the determination of telomerase activity, notably in such samples, is the risk of false-negative or false-positive results. Telomerase activity might not be found in a cancer sampledue to incomplete sampling or due to suboptimal preservation of the sample. On the other hand, telomerase activity can also be found in a sample without cancer cells when telomerase-positive stem cells or inflammatory cells are present. Nevertheless, the TRAP assay for telomerase activity may has potential as a screening tool. It is important to determine whether telomerase can be measured correctly in samples obtained from patients using non or minimally invasive procedures. The use of telomerase assay in minimally invasive procedures, such as cytological diagnosis for pleural or peritoneal effusions, may hold great promise for aiding clinical diagnosis prior to surgery and in some instances, in which the cytology is not informative, may help differentiate benign from malignant effusions.

1.1.3.3 Telomerase activity in soft tissue sarcomas and the alternative mechanisms for lengthening of telomeres

Schneider-Stock found that telomeric lengths vary in soft tissue sarcomas (Schneider-Stock et al, 1998); telomerase was activated in only 69% of liposarcomas (Schneider-Stock et al, 1999). No data was presented concerning telomerase activity in other types of soft tissue sarcomas. Clearly, sarcomas need to be studied more closely. The available data suggest, however, that immortalization of mesenchymal cells might imply different mechanism than immortalization in epithelial cells. Immortalization of human cells in culture is usually associated with telomerase activation. However, some human cell lines immortalised *in vitro* have been found to have no detectable telomerase activity, such as IHCF-T/A6 (fibroblast derived) and BET-3M (bronchial epithelial cell derived) (Bryan et al, 1995). Mixing experiments showed that inhibitors of telomerase or of the TRAP assay are not responsible for these negative results. The

extreme heterogeneity in telomere length, and the differences in the dynamics of telomere maintenance in telomerase-negative cell lines compared to telomerase-positive cell lines, indicate that these cells might have utilised one or more alternative mechanisms for lengthening of telomeres (ALT) (Bryan et al, 1995; Henderson et al, 1996; Lansdorp et al, 1997). ALT is not restricted to cell lines immortalised *in vitro*, but has also been found in 4 of 56 tumour-derived cell lines. 3 of these 4 ALT cell lines were derived from sarcomas (Reddel et al, 1997). Bryan and co-workers discovered that immortalised cell lines derived *in vitro* from fibroblasts were more frequently ALT-positive than immortalised cell lines derived *in vitro* from epithelial cells (Bryan et al, 1997). More recently, Yeager et al. reported that ALT cells and tumors contain a novel promyelocytic leukemia (PML) body (ALT-associated PML body, APB) in which PML protein colocalized with telomeric DNA and the telomere binding protein hTRF1 and hTRF2. APBs were found in ALT tumors and cell lines but not in mortal cell strains or in telomerase-positive cell lines or tumors (Yeager et al, 1999).

1.1.4 Perspectives

The discovery of telomerase has provided us a potentially powerful new marker and prognostic indicator as well as a target for new cancer therapies. The field is moving rapidly and in a limited time frame, much has been discovered concerning the structure and function of telomerase, but it is a still relatively new area and much further work is needed to answer following questions.

(1) How is telomerase activity regulated?

(2) Are there alternative telomere lengthening mechanism?

(3) Can a telomerase assay be used as a diagnostic and prognostic marker in neoplastic lesions?

(4) Are telomerase blocking reagent potentially valuable new therapeutic approaches?

1.1.4.1 Regulation of telomerase activity

Cancer is essentially a disease invoving dynamic changes in the genome. The foundation for this concept has been set in the discovery of mutations that produce oncogenes with dominant gain of fuction and tumour suppressor genes with recessive

loss of function. The expression of telomerase activity is likely regulated by various oncogenes and tumour suppressor genes, both directly and indirectly. The mechanism of regulation of telomerase is currently focused on hTERT gene. Sequence analysis has revealed that the hTERT promoter contains binding sites for several transcription factors suggesting that hTERT gene expression might be regulated by different factors (Cong et al, 1999; Wick et al, 1999). Recent studies shown that the proto-oncogene c-Myc activates telomerase by inducing expression of its catalytic subunit (Wang et al, 1988; Wu et al, 1999). Introduction of chromosome 3 into telomerase-positive cell lines, human renal carcinoma (Horikawa et al, 1998; Tanaka et al, 1998) and breast cancer cells (Cuthbert et al, 1999) induces repression of hTERT expression. A putative telomerase repressor gene has been further mapped to chromosome region 3p14.3-p21.3 (Tanaka et al, 1998; Parkinson et al, 1997). In addition, up-regulation of telomerase is associated with introduction of HPV-16 E6 protein (Klingelhutz et al, 1996; Kiyono et al, 1998), SV40 , v-K-ras (Burger et al, 1998; Rhim et al, 1998) and Bcl-2 (Mandal et al, 1997) and downregulation with introduction or overexpression of retinoblastoma protein (Xu et al, 1997; Nguyen et al, 1999). Taken together, activation of telomerase might be regulated by many factors at multiple level.

More recently DNA methylation has been found to play a role in gene silencing (Herman et al,1994) and as a mechanism for the loss of function of tumour suppressor gene in the onset or the progression of cancer (Sakai et al, 1991; Herman, 1999; Baylin et al, 1998). Hypomethylation of DNA is associated with increased gene expression (Ferguson et al, 1995), and might be involved in activation of oncogenes. Gene transcription can be activated by treatment with demethylating agents (Venolia et al, 1983). Therefore, DNA methylation of cytosine in CpG sites of promoter regions is considered the most common mechanism for repressing of gene expression. Even a unique methylated CpG site in the promoter region is sufficient for a significant down regulation of *in vivo* transcription. Based on these consideration, we propose that DNA methylation might play a role in the regulation of telomerase activity. Future In depth is needed to analyse to what extent DNA methylation in the promoter regions of hTR and hTERT genes, plays a role in the (de)activation of telomerase.

1.1.4.2 Alternative mechanism(s) for maintaining telomere length

Immortalization of human cells in culture is usually associated with telomerase activity. In some cases, however, telomerase appears not to be activated. Heterogeneity of telomere length has been found between chromosomes in individual telomerase-negative cells (Henderson et al, 1996; Landsdorp et al, 1997) and between telomerase-negative human tumours (Schneider-Stock et al, 1998; 1999). Long telomeres suggest that telomere lengthening has occurred. Extreme heterogeneity in telomere length in telomerase-negative cells suggest that they must have utilised one or more alternative mechanisms of telomere elogation (ALT). The nature of the ALT mechanism(s) in human is currently unknown, eukayotes use two other mechanisms for maintenance of telomere length: recombination (Wang and Zakian, 1990) and retrotransposition (Moore and Haber, 1996; Teng et al, 1996). A detailed understanding of ALT will be necessary in order to increase efficiency of cancer therapy by telomerase inhibitors. Much further study is needed, even though ALT appears to be present in only a minority of tumours. The ALT-associated PML body (APB) provides a simple marker for ALT. Soft tissue sarcomas constitute an interesting model to study the ALT mechanism(s) in human tissues.

1.1.4.3 Telomerase in diagnosis and prognosis of neoplasia

Many publications agree that telomerase activation can be used as a criterion to diagnose neoplasia of the colon, head and neck, lung, and skin, even at a preinvasive stage. Future studies are needed to determine the value of telomerase activity and the expression of its components (hTR and hTERT) for (early) cancer diagnosis.

Other studies revealed that telomerase activity is associated with clinical outcome in certain cancers. Telomerase activation has been shown to correlate with poor clinical outcome in gastric cancer (Tahara E et al, 1996), breast cancer (Kim et al, 1994), ordinary minigioma (Longford et al, 1997) and neuroblastoma (Hiyama et al, 1995). Telomerase activity has been also found to correlate with pathological stage (Tahara et al, 1995) or tumour aggressiveness (Ohyashiki et al, 1997; Hoos et al, 1998). Futhermore, the level of telomerase correlates with survival rates of patients (Hiyama E et al, 1995; Langford et al, 1997). Future studies should focus on distinguishing the pre-cancerous lesions that will likely progress to invasive cancers

from the ones that will not, predicting the clinical course of a patient after the cancer has been diagnosed, and monitoring the effectiveness of cancer therapy. For some types of cancer, telomerase might be one such marker.

1.1.4.4 Telomerase in cancer therapy

Successful cancer therapy requires agents that efficiently and specifically kill tumour cells but not normal cells. Because telomerase activity is high in a wide variety of human cancers and absent in most normal cells, it might be a plausible target for new cancer therapies. Telomerase inhibitors have recently been considered as potential antitumour agents. In general, the potential target for inhibiting telomerase activity might include: the hTERT active site; the 11-base RNA template; the 'anchor site' where hTERT interacts with telomeric DNA; the extended telomere, possibly at a G-quadruplex structure; interacting proteins; or antisense targeting of the mRNA for either hTR or hTERT (Pitts and Corey, 1999). There have been several reports on the inhibition of telomerase activity in human cells, including microcell-mediated chromosome fusion (Ohmura et al, 1995), antisense hTR-based approaches (Feng et al, 1995 and Bisoffi et al, 1998), reverse transcriptase inhibitors (Strahl and Blackburn, 1996), and a telomere-binding protein (Broccoli et al, 1997). Although some of those approaches effectively inhibit telomerase activity, it is still too early for clinical application. Many problems remain to be resolved as, for example, how to taget telomerase inhibitors specifically to tumour. In addition, the proposed alternative pathway(s) used by immortal cells to maintain their telomere might be a limiting factor. The key to designing or choosing drugs that will specifically attack telomerase lies in understanding the structure and the mechanism of regulation of telomerase.

1.2 Outline of the present study

Our studies were undertaken to answer the following questions: (1) At which stage in colorectal carcinogenesis is telomerase activated? (2) What is the relationship between telomerase activity and the expression of hTR RNA and hTERT mRNA? (3) Is telomerase activated in soft tissue lesions? (4) Can the telomerase assay be used in addition to cytological examination in the detection of neoplastic cells in effusions?

In **chapter 2,** a methodological validation of our TRAP assay is reported. As a ribonucleoprotein enzyme, telomerase needs intact RNA as a template and an active protein moiety for the reverse transcription of the telomeric TTAGGG repeats. These two conditions indicate that the quality of the tissue is of vital importance for a reliable telomerase assay. We use rRNA integrity as a control for the quality of tissue preservation.

In **chapter 3,** we determined at which morphologically defined step during human colorectal carcinogenesis telomerase is activated. In order to establish telomerase activation relative to molecular genetic events, K-*ras* and *p53* mutations were assayed in the same material.

In **chapter 4,** we studied the expression of the telomerase genes (hTR RNA and hTERT mRNA) and correlated these with telomerase activity in human colorectal carcinogenesis. Absence of intron sequences is one of the particularities of the hTR gene, and the problem of contamination of extracted RNA by genomic DNA for hTR RNA analysis by RT-PCR was solved by extensive DNase digestion.

In **chapter 5,** Telomerase activity has been detected in a majority of human carcinomas, but little is known regarding soft tissue sarcomas. We determined telomerase activity and hTERT mRNA in a series of soft tissue lesions. Since telomerase activation correlates with proliferative activity of cells, we also examined this in sarcomas using Ki-67 immunolabelling.

In **chapter 6,** we tested whether the presence of telomerase activity correlates with cytology status in effusions.

In **chapter 7,** our findings are discussed in the context of the current literature.

1.3 References

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CHAPTER II

Tissue quality is an important determinant of telomerase activity as measured by the TRAP assay

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ABSTRACT

Almost without exception, cancer cell lines have telomerase activity, which does not seem to be the cases for cancer tissues. A possible reason for this apparent discrepancy could be false-negative results, due to quality of tissue samples. To test this hypothesis, we analyzed frozen tissue specimens of 50 colon cancers for telomerase activity. As RNAs are very easily degradable molecules, we used RNA degradation as a parameter of the quality of the tissues. We observed that when the RNA was not or only partially degraded in all (25 cases) telomerase activity was detected. In contrast, when RNA was highly degraded, 32% of cases (8/25) showed no telomerase activity. We propose that RNA quality control is evaluated as a routine in the TRAP assay.

Telomerase is a ribonucleoprotein with the function of a DNA polymerase, in which a segment of the RNA component operates as an internal template. It adds hexameric (TTAGGG) repeats to the telomeric ends of the chromosomes, thus compensating for the continued erosion of the telomeres that occurs in its absence (3,7). Reactivation of telomerase may be a necessary event for the sustained growth of most human tumors (9). The development of a PCR-based telomerase assay - the telomeric repeat amplification protocol (TRAP) - has permitted a large number of tumor samples to be analyzed (5).

Telomerase activity has been detected in almost all human immortal cell lines and in the great majority of human cancer tissues (2,5). Some human tumor tissues, however seem not to express telomerase. A review which compiled several studies of human tumors indicated that telomerase activity occurred in human cancer tissues in between 75% and 100% of cases, with an average of 84.8% (8). Furthermore, different studies on histologically similar tumors revealed different proportions of tumors with telomerase activity. For example, in colorectal cancer telomerase activity was detected in 32 of 35 (92%) by Kim et al. (5), in 40 of 50 (80%) by Li et al. (6), and in 8 of 8 (100%) by Yoshida et al. (11). An explanation for these discrepancies could be heterogeneity of human tumor tissues : these invariably consist of mixtures of tumor

cells and surrounding stromal tissue. However, the TRAP assay has been shown to detect telomerase activity in as few as 1 positive cell per 10^4 cells (5). We propose that some of the apparently negative tumors yield RNA of insufficient quality and are therefore false-negative.

A second reason for false-negative results might be the presence of Taq DNA polymerase inhibitors. To recognize this type of problem, the TRAP assay should be performed using an internal control (10). The TRAPeze kit (Oncor Inc., Gaithersburg, MD) includes the amplification of an internal control of 36 bp in each assay, and a false-negative result is concluded when the 36 bp amplified product is not observed. This control, however, does not account for all apparently negative tumor tissues.

Telomerase is a ribonucleoprotein enzyme that uses its RNA as a template for the synthesis of TTAGGG repeats at the ends of the chromosomes (4). For this reason, telomerase activity necessitates an active protein and a non-degraded RNA. These two conditions indicate that the quality of the tissue is of vital importance for the success of the telomerase detection assay. Strikingly, RNA quality control has never been proposed to date. We therefore included a control for RNA quality in our TRAP assay protocol.

We measured telomerase activity in 50 colorectal cancer tissues derived from 50 patients using the TRAP assay (Oncor). About 10 mg of frozen tissue was homogenized in 150 µl of 1×CHAPS lysis buffer. The whole tissue lysate was rapidly frozen and stored at -80°C. The protein concentrations of the extract were measured using the BCA protein assay kit (Pierce Chemical Corp., Rockford, Illinois, USA). About 0.1 µg of protein extracted from tissue was used for each telomerase assay (25 µl reaction). Forty two samples (84%) were found to be positive in this assay, while 8 samples were telomerase negative. All 50 samples were taken from the tissue bank of the Institute of Pathology of Lausanne. Some samples were not frozen immediately, a relatively important lapse of time occurred sometimes between surgery and freezing in liquid nitrogen. Warm ischemia might account for tissue autolysis including RNA degradation, which may affect the telomerase activity results. To test this hypothesis, total RNA from these 50 colon cancer tissues was extracted using Trizol (Life Technologies). For each case about 50 mg of frozen tissue was used to extract RNA. The quality of RNA was controlled on agarose gels (Figure 1A). In 25 out of 50 colon

cancer tissues, the RNA showed no or partial 28S and 18S ribosomal RNA degradation (Figure 1A, lanes 3 to 6). In the other 25 cases significant to complete RNA degradation was observed (Figure 1A, lanes 7 to 9). All of the 25 cases with only partially degraded or intact RNA showed telomerase activity (Figure 1A and 1C, lanes 3 to 6). Only 17 of the 25 (68%) cases with strongly or complete degraded RNA were positive for telomerase activity (Figure 1A and 1C, lanes 7 to 9). This difference is statistically significant ($p<0.0001$). Various levels of telomerase activity was observed. All the 25 cases with only weak RNA degradation showed strong telomerase activity (Fig 1C, lanes 3 to 6), whereas a majority of cases with strongly degraded RNA had either weak (Figure 1C, lanes 7 and 8) or even no (Figure 1C, lanes 9 and 10) telomerase activity. Almost all the cases with complete RNA degradation had no detectable telomerase activity (Figure 1A and 1C, lanes 9 and 10). The fact that telomerase activity was observed in some cases with apparent RNA degradation could be explained on the assumption that full length of the RNA component of telomerase is not necessary for telomerase activity (1). Another explanation for the presence of telomerase activity in partially degraded samples could be the sensitivity of the TRAP assay. Indeed, even an important reduction in the number of active telomerase molecules due to tissue degradation may not totally abolish detectable telomerase activity in the sample.

A limitation of this approach is that the amount of tissue required to perform an rRNA measurement is too high for small biopsies. To overcome this limitation, different approaches have been tested. First, RNA quality was determined directly from the RNA in tissue extracts obtained from CHAPS lysis buffer. Using Trizol, 5-10 µg of total RNA was obtained from tissue extracts (60 µg of protein extract). Some rRNA degradation was observed by agarose gel analysis, and in a majority of cases no 28S and 18S ribosomal RNA bands were observed. The same RNAs were also analyzed by RT-PCR using β-actin and GAPDH as markers. Almost all cases gave an amplification of β-actin and GAPDH, even in the cases where telomerase activity was undetectable. RT-PCR gave many false-positive results and is therefore not a suitable method to determine tissue quality. In another approach, total RNA was extracted from consecutive tissue sections. Depending on the size of the tissue sample to analyze, 1-5 sections of 10 µM are enough to extract cells in 30 µl of CHAPS lysis buffer for

telomerase analysis, whereas 4-10 sections are necessary for extraction of a sufficient amount of RNA (total RNA dissolved in 20 µl water). Using this approach, the results for telomerase activity and rRNA measurements are perfectly superimposable with those obtained with large amount of tissues (Figure 1A and 1B for rRNA quality). Furthermore, sections for histological analysis can be prepared just before and after the section necessary for telomerase and RNA analysis. Thus, histological characteristics of the analyzed lesions can be correlated directly to telomerase activity and false-negative results due to sampling error can be avoided.

These results indicate that if RNA is not strongly or completely degraded (28S and 18S ribosomal RNAs species not completely lacking) more reliable results will be obtained. If RNA is strongly degraded, telomerase activity is not always observed (Table 1). Our results clearly indicate that for a valid TRAP assay, the control of RNA quality is essential in order to reduce the number of false-negative results. This control is simple and rapid (can be done in less than 2 hours).

Table 1. Telomerase activity and RNA degradation in olorectal cancer tissues

	RNAi	RNAd
Telomerase+	25	17
Telomerase -	0	8

RNAi: RNA intact or only partially degraded

RNAd: high RNA degradation (28S and 18S ribosomal RNAs species completely lacking)

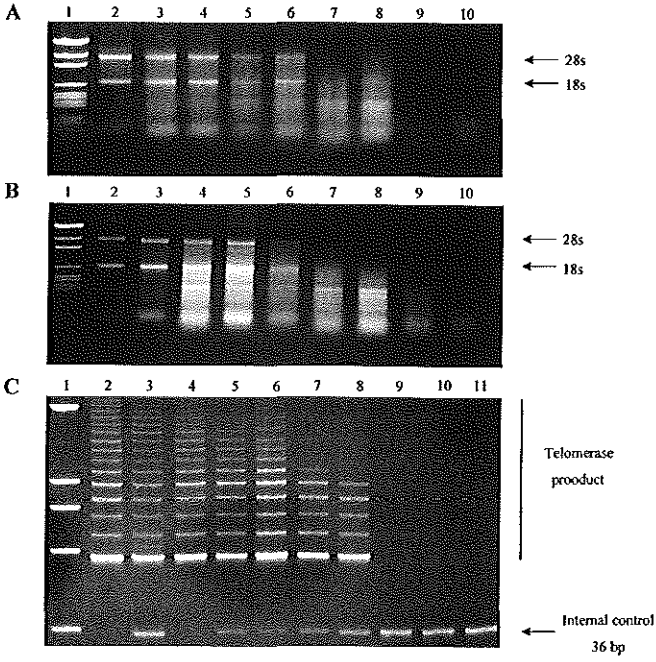

Figure 1. RNA quality and telomerase activity in colorectal cancer tissues. (A+B) Total RNA extracted from frozen colon cancer tissues (A) and from tissue sections (B) was subjected to 1% agarose gel electrophroresis. Lanes 2 to 10 contained about 0.6-1.0 μg of total RNA.. (C) Telomerase activity was detected using the TRAP assay from Oncor. About 0.1 μg of protein extracted from frozen colon cancer tissues was placed in 25 μl of PCR reaction mixture for each assay. One of fifth of PCR amplification product was applied to a 12.5% non-denaturing polyacrylamide gel electrophoresis and visualized by SYBR green I staining. Lane 1: pGEM DNA marker (Promega). RNA analysis (part A+B) or telomerase assay (part C) from SW480 colorectal cancer cell line (lane 2) or from frozen colorectal tumor tissues (lanes 3-10). Lane11, part C: negative control where no protein was added.

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CHAPTER III

Telomerase activation in colorectal carcinogenesis

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SUMMARY

Telomerase activity has been detected in germ cells as well as in the developing embryo. Activity is no longer detectable in most somatic cells of the neonate, although low levels of activity persist in regenerative tissues. Telomerase has been found to be reactivated or upregulated in a majority of cancers. The colorectal adenoma-carcinoma sequence is one of the best characterised models of multistep tumorigenesis, and thus suitable to determine at which stage telomerase is activated. Telomerase activity was examined by telomeric repeat amplification protocol (TRAP) assay in 96 cases of colorectal tissues, including 50 carcinomas, 31 adenomas and 15 normal colon tissues. For each case, histological diagnosis and telomerase activity were determined on consecutive frozen sections. In order to reduce the chance of a false-negative TRAP assay due to RNA degradation, the integrity of rRNA in the tissues was verified in each case. Twenty five carcinomas, 30 adenomas and all of the 15 normal colorectal mucosa samples showed no or only partial rRNA degradation and only in these cases the TRAP assay was interpreted. None of the normal tissues exhibited telomerase activity. In contrast, all of the 25 cancers and 47% (14/30) of the adenomas were positive. In adenomas, telomerase activation was highly significantly related to grade of dysplasia ($P<0.0001$). All adenomas which contained HG dysplasia revealed telomerase activity, whereas in only 20% (4/20) of cases with exclusively LG dysplasia telomerase activity was detectable. Our results indicate that telomerase activation, which might be an obligatory step in colorectal carcinogenesis, occurs in the progression from low grade to high grade dysplasia in adenoma. Furthermore, in the adenoma-carcinoma sequence telomerase activation seems to occur later than K-*ras* mutation but earlier than *p53* mutation.

Key words: Telomerase, TRAP assay, Colorectal carcinoma, Colorectal adenoma, K-*ras* mutation, *p53* mutation

INTRODUCTION

The telomere-telomerase hypothesis of cell aging has rapidly become a focus of intensive research in the field of carcinogenesis. The hypothesis is based on the observation that telomerase activity is expressed in embryonic cells, in adult germline cells (1, 2), and in most human tumours (1, 3), but is undetectable in normal somatic tissues except for proliferative cells of renewable tissues including haemopoietic stem cells (4, 5, 6), activated lymphocytes (4), basal cells of the epidermis (7, 8) and intestinal crypt cells (9). Human telomeres, which are composed of many kilobases of TTAGGG repeats and associated proteins, protect and stabilize chromosome ends during DNA replication (10, 11). In normal somatic cells telomeric DNA is lost with each cell division, because DNA polymerases synthesise DNA in the 5' to 3' direction and require an RNA primer for initiation. The end replication problem (12) predicts the progressive loss of telomeric DNA. Thus, telomere reduction is thought to be a biologic clock regulating the life span of a cell. In contrast to normal cells, tumour cells show no loss of average telomere length with cell division, suggesting that telomere stability may be required for cells to escape from replicative senescence, and thus can proliferate indefinitely. This telomere length stability appears to be the consequence of telomerase activation. It is likely a necessary event for sustained cell growth in malignant tumours. Telomerase, a ribonucleoprotein complex, is a specialised reverse transcriptase that synthesises telomeric DNA sequences and thus restores telomere length (13,14).

Telomerase activity has been found in almost all human cancer tissues and cancer cell lines tested to date (15). However, at which moment in the multistep pathogenesis of cancer telomerase is activated remains to be clarified. If telomerase could be used to detect preinvasive neoplasia, this could then be treated with greater efficiency and lesser toxicity than invasive cancer. So, early detection based on telomerase activation assays might have the potential to reduce cancer morbidity and mortality.

The colorectal adenoma-carcinoma sequence is one of the best characterised models for multistep tumorigenesis. Progression from adenoma to carcinoma has been shown to occur along with stepwise accumulation of distinct genetic alterations, each

new alteration contributing to progression of the lesion through clonal expansion of a cell with a new alteration which confers a growth advantage (16). Tissue samples from different stages can be obtained relatively easily which allows characterisation of the histopathological steps and accompanying genetic alterations. Telomerase activity has been found in 90-95% of stage I colon carcinomas, but at present its presence in adenomas has not been unambiguously established. Some authors have indicated that telomerase is activated in colorectal adenomas, but at a low level (17). Others have reported absence of telomerase activity in colorectal adenomas (1, 18). As pointed out by Meeker and Coffey (19), there are many pitfalls in assaying telomerase activity, including inadequate handing of samples, sampling artefact, contamination with active material and PCR inhibitors. For this reason, it is quite important to use proper positive and negative controls. Another reason for conflicting results, could be the occurrence of heterogeneity within colorectal adenomas. In a previous study, we have shown that genetic and histopathologic heterogeneity occurs in a great majority of colorectal adenomas (20). This finding underlines that pathological characteristics and telomerase activity should be compared on several sections taken from the same tissue sample, to allow for adequate histological information.

The aim of our study was to determine at which morphological steps during human colorectal carcinogenesis telomerase is activated under experimental conditions allowing an appreciation of RNA integrity and assessment of the degree of dysplasia in the tissue fragment assayed for telomerase. Telomerase activity was detected by the TRAP (telomeric repeat amplification protocol) assay (21); which is a sensitive, reliable and reproducible test, when suitable controls are included (19). In order to established telomerase activation relative to molecular genetic events, K-*ras* and *p53* mutations were assayed in the same material.

MATERIALS AND METHODS

Patients and Tissue Samples

Tissue samples from 50 surgically resected colorectal carcinomas were obtained from the tissue bank of the Institute of Pathology of Lausanne. Thirty colorectal adenomas were obtained by colonoscopic polypectomy. The size of polyps was measured immediately upon reception of the samples. Peripheral slices of the polyps were immediately embedded in OCT, frozen in liquid nitrogen, and stored at -80°C until use. From 15 non-cancer related colectomy cases, apparently normal colon mucosa was sampled and frozen as described.

Sample Preparation

From each colorectal case, a thin piece of tissue was sampled for DNA extraction. Then, 10-15 consecutive cryostat sections were cut. The first and the last sections were H&E stained in order to assess histological characteristics. The sections in between were cut at 12 µm and put into two chilled 1.5 ml Eppendorf tubes using a tapered glass pipette. Depending on the size of the tissue sample, 1-5 sections were sufficient to extract protein in 30 µl of CHAPS lysis buffer for the detection of telomerase activity, and 5-10 sections were necessary for extraction of a sufficient amount of total RNA.

Histological assessment of dysplasia in the sample

H&E stained cryostat sections were reviewed by two pathologists (ES, HB). For histological diagnosis and grading of dysplasia, the criteria of the World health organisation (23) and of the National Polyp Study Group (24) were used. According to the degree of dysplasia, adenomas were grouped into low-grade (mild and moderate) and high-grade (severe dysplasia and in situ carcinoma, confined to the mucosa) categories. The grade of dysplasia was established according to the most severely dysplastic area. Furthermore, the proportion of the high-grade dysplasia (HG) was established by semi-quantitative scoring and this allowed us to classify the cases into three groups : (LG) adenomas with low grade dysplasia only; (LG+HG) adenomas with low grade and high grade dysplasia, when HG was present in 1-50% of the

section; (HG) adenomas with high proportion of high grade dysplasia, when HG dysplasia was present in more than 50% of the sample.

Control of RNA degradation

Total RNA was extracted from tissue sections using Trizol (Life Technology), and after ethanol precipitation in presence of 10μg glycogen redissolved in 20 μl of RNase-free water. About 0.6 -1.0 μg of total RNA was subjected to 1% agarose gel electrophoresis. When 28S and/or 18S ribosomal RNA (rRNA) bands appeared on the agarose gel, rRNA was judged intact or partially degraded. If no 28S and 18S rRNA bands were present, rRNA was judged totally degraded (22).

TRAP assay for Telomerase Activity

Telomerase activity was determined using the TRAP assay according to manufacturer's protocol of the TRAPeze Telomerase Detection Kit (Oncor). For the colorectal carcinoma cell line SW480, cell pellets (about 1×10^5 cells) were suspended with 400 μl of CHAPS lysis buffer. For frozen tissues, 1-5 sections were homogenised with 30 μl of CHAPS lysis buffer. After incubation for 30 min on ice, the lysate was centrifuged and the supernatant was stored at -80°C immediately. Protein concentration of the extract was measured by the BCA protein assay kit (Pierce Chemical Corp., Rockford, Illinois, USA). For each case the TRAP assay was performed in three different concentrations of protein extracts: 0.1, 0.5 and 1 μg. The TRAP reactions (25 μl final volume) contained 1×TRAP buffer , 50 mM dNTPs, 0.5 μl of TS primer, 0.5 μl of TRAP primer mix, 1 unit *Taq* DNA polymerase (Boehringer Mannheim) and an aliquot of CHAPS cell extract (1, 0.5 or 0.1 μg of protein extract). The extract of colorectal carcinoma cell lines (equivalent to 250 cells) was used as a positive control in the telomerase assay. TRAP reaction mixture was placed in a thermocycler and incubated for 30 min at 30°C, 94°C for 2min, then 32 cycles of 94°C for 30 s, 56°C for 45 s and 72°C for 45 s were performed. PCR products (5 μl) were electrophoresed on a 12.5% polyacrylamide nondenaturing gels. The gels were stained with SYBY green I (FMC Bio Products) and visualised under UV light using a CCD camera.

Detection of K-ras and p53 Mutations by Non-radioactive Single Strand Conformation Polymorphism (SSCP)

Genomic DNA was extracted from samples using standard methods. Exon 1 of K-*ras* and exons 5 to 9 of *p53* genes were amplified by PCR. The PCR reaction was carried out for 35 cycles using the following amplification profile: denaturation at 94°C for 30 sec, annealing at 54-60°C for 45 sec, and extension at 72°C for 45 sec. Correct amplification was controlled by electrophoresis on a 2% agarose gel. Five to forty ng of PCR product were then analysed by non-radioactive SSCP as described previously (20,25).

RESULTS

Telomerase Activity in colorectal tissues

By analysis of ribosomal RNAs (rRNA), we found that only 25 of the 50 colorectal cancer tissues had intact or only partially degraded rRNA, the other 25 cases showing highly degraded rRNA. This is most likely due to the surgical procedure, in which the early ligation of the perfusing vasculature might be responsible for a relatively long preoperative warm ischemic period. This explanation is consistent with the fact that only one adenoma and no normal colorectal mucosa samples showed rRNA degradation. Amplification using the TRAP-eze kit resulted in the formation of a 36-bp band in every lane (Fig. 1), which served as an internal control to identify false negatives due to the presence of Taq polymerase inhibitors. In our series, all samples demonstrated a 36-bp band. All of the 25 cases of colorectal cancer with only partially degraded or intact rRNA contained telomerase activity. Only 14 out of 30 (47%) cases of adenoma, with no or only very partially degraded rRNA, expressed telomerase activity. No telomerase activity was found in 15 normal colon mucosa samples (Table 1).

Table 1. Telomerase activity in colorectal tissues

	No of positive cases/ No of cases	% positive
Colorectal carcinoma	25/25	100
Colorectal adenoma	14/30	47
Normal colorectal mucosa	0/15	0

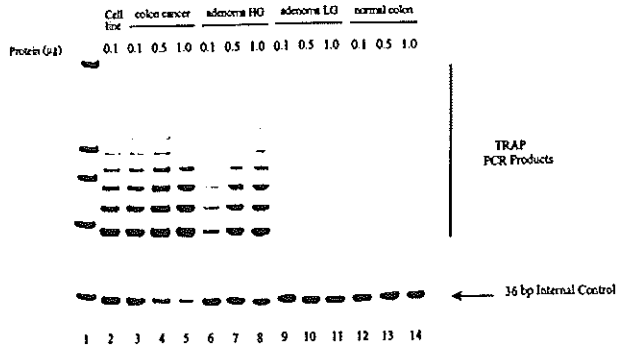

Figure 1: Telomerase activity in colorectal tissues. From each tissue sample, telomerase assays were performed by the TRAP method using extracts containing 0.1, 0.5 or 1 μg of protein (lanes 3-14). The extract of the human colorectal carcinoma cell line SW480 (equivalent to 250 cells) was used as a positive control (lane 2). The 36-bp internal positive control band was seen in every lane, and it was used to identify non-informative specimens due to inhibitors of *Taq* polymerase. A size marker, pGEM DNA marker (Promega), was also shown in lane 1.

Correlation with Histopatological Characteristics and Telomerase activity in colorectal adenomas

Telomerase activity was detected in 35% (7/20) of adenomas with a size smaller than 2 cm and in 70% (7/10) of adenomas larger than 2 cm; but this difference is not statistically significant. No correlation was observed between the presence of telomerase activity and architecture of the adenomas (Table 2).

Histopathological analysis of the adenomas, showed LG dysplasia (LG) in 20 cases, less than 50% HG dysplasia (LG+HG) in 4 cases, and more than 50% HG dysplasia (HG) in 6 cases. Telomerase activity was significantly correlated with the grade of dysplasia in the assayed tissue sample ($P<0.0001$). Telomerase activity was present in all of the adenomas with HG dysplasia (10 cases) and in only 20% (4/20) of the adenomas which did not contain HG dysplasia (Table 2).

Table 2. Correlation between telomerase activity, K-ras mutation and histology in colorectal adenomas

	Telomerase positive (%)	Telomerase negative (%)	No of cases	p^a
Size:				
0.8 - 2 cm	7 (35%)	13 (65%)	20	0.07
> 2 cm	7 (70%)	3 (30%)	10	
Histology				
Tubular	7 (54%)	6 (46%)	13	
Tubulo-villous	5 (38%)	8 (62%)	13	0.72
Villous	2 (50%)	2 (50%)	4	
Dysplasia [b]				
LG	4 (20%)	16 (80%)	20	<0.0001
HG	10 (100%)	0	10	
K-*ras* mutation				
Negative	6 (38%)	10 (62%)	16	0.28
Positive	8 (57%)	6 (43%)	14	
Total cases	14 (47%)	16 (53%)	30	

[a] chi-square test.

[b] LG: low grade dysplasia only; HG: high grade dysplasia. Grade of dysplasia was established in the same sample where telomerase activity was assessed.

One HG sample was devoid of detectable telomerase activity by the standard TRAP assay (Table 3, case 21). To elucidate whether absence of telomerase activity was not due to degradation of telomerase RNA component, a ribonuclease inhibitor was added during the process of protein cell extraction. The ribonuclease inhibitor (RNasin from Promega, Madison WI) was used at a concentration of 0.3 U per µl of

CHAPS lysis buffer. As expected, a weak telomerase activity was recovered. Telomerase negative adenomas with only LG dysplasia were also tested for presence of RNase, but none of them revealed telomerase activity even when RNasin was previously added to the cell extract.

Telomerase activity, K-ras and p53 mutations in colorectal adenomas and carcinomas

In 14 of 30 (47%) adenomas a K-*ras* mutation was observed in the same area of the adenoma where telomerase activity was measured (Table 2 and 3). In our series, the proportion of adenomas and carcinomas with a K-*ras* mutation were very similar, 47% and 48% respectively. The frequency of K-*ras* mutations seems to increase with either the grade of dysplasia or presence of telomerase activity (Table 3). Six of 10 (60%) of adenoma samples with HG dysplasia but only 8 of 20 (40%) adenoma samples with LG dysplasia harboured a K-*ras* mutation, but this difference did not attain statistical significance. In the same manner, no positive correlation between increased telomerase activity and K-*ras* mutation has been observed. As expected, *p53* mutations were more predominant in cancers than in adenomas, respectively 40% and 10%. Only 3 (10%) adenomas harboured a *p53* mutations and this genetic alteration was observed in low grade as well as in high grade adenomas (Table 3).

Table 3. Telomerase activity, histological characteristics and genetic alterations in colorectal adenoma

Case No	Size (cm)	Type[a]	Grade of dysplasia	%HG dysplasia	Telomerase activity[c]	K-*ras*[d]	*p53*[d]
1	<1	V	LG	0	–	wt	wt
2	<1	T	LG	0	+	12-GAT	wt
3	1-2	T-V	LG	0	–	wt	wt
4	1-2	T-V	LG	0	–	wt	wt
5	1-2	T	LG	0	–	12-GAT	wt
6	<1	T	LG	0	+	12-GAT	wt
7	<1	T	LG	0	–	wt	wt
8	<1	T-V	LG	0	–	wt	exon 8
9	1-2	T-V	LG	0	–	wt	wt
10	>2	V	LG	0	+	wt	wt
11	>2	T-V	LG	0	–	12-GAT	wt
12	>2	T-V	LG	0	+	wt	wt
13	>2	T-V	LG	0	–	12-GAT	wt
14	1-2	T-V	LG	0	–	wt	wt
15	1-2	T	LG	0	–	wt	wt
16	1-2	T	LG	0	–	12-AGT	wt
17	>2	V	LG	0	–	12-GAT	wt
18	1-2	T	LG	0	–	12-GAT	wt
19	1-2	T-V	LG	0	–	wt	exon 8
20	<1	T	LG	0	–	wt	wt
21	<1	T	LG+HG	3	+[e]	wt	wt
22	1-2	T	LG+HG	10	+	13-CGC	wt
23	>2	T-V	LG+HG	10	+	wt	wt
24	>2	T-V	LG+HG	10	+	wt	wt
25	1-2	T	HG	60	+	w	wt
26	>2	V	HG	60	+	12-TGT	wt
27	>2	T-V	HG	80	+	12-AGT	exon 7
28	1-2	T	HG	80	+	12-GTT	wt
29	1-2	T-V	HG	80	+	13-GAC	wt
30	>2	T	HG	90	+	13-GAC	wt

[a] T= tubullous adenoma ; T-V = tubullo-villous adenoma ; V = villous adenoma
[b] LG, low grade dysplasia; LG+ HG, low grade dysplasia with <50% of high grade dysplasia ; HG, ≥50% high grade dysplasia.
[c] Telomerase activity assayed by TRAP.
[d] K-*ras* and *p53* mutations detected by SSCP.
[e] Positive by TRAP only when a ribonuclease inhibitor was added during cell extract preparation.

DISCUSSION

Telomerase activity is found to be activated or upregulated in a majority of human cancers (15,19). There is some evidence that telomerase activity is present early in some specific cancers (26-30), but conflicting results have been reported in colorectal tumorigenesis (17,18). To date, it is still unclear at which stage of colorectal carcinogenesis telomerase is activated and whether or not telomerase activation might be indicative of malignant potential in a colorectal adenoma. We therefore studied telomerase activity in colorectal carcinomas (25 cases selected for intact rRNA), colorectal adenomas (30 cases) and normal colon mucosa (15 cases). We found that all carcinomas and 47% (14/30) of the adenomas expressed the enzyme; telomerase activity was not detected in normal colon tissues.

Telomerase activity has been detected in almost all human cancer cell lines and in the vast majority of human cancer tissues (15). Nevertheless, in about 10%-15% of the reported human cancers telomerase activity was not detected (1). In principle, for seemingly telomerase negative cases several explanation should be considered. Firstly, telomerase independent mechanisms for the conservation of telomere length should be considered (31). These most probably exist but this remains elusive as yet. Secondly, telomerase might remain undetectable due to technical problems such as polymerase inhibitors or degradation of the enzyme complex. The latter problem has been overlooked but appears to be quite important. Of our colorectal cancer samples with undegraded rRNA 100% (25 cases) were telomerase positive, whereas only 64% (16 of 25 cases) of the samples with degraded rRNA showed telomerase activity. This difference is statistically significant ($P<0.0001$). This observation clearly indicates that the integrity of RNA is an important determinant of the TRAP assay results. In fact, even slight degradation of the cell sample could lead to inactivation of the telomerase complex. Degradation of RNA is most likely not a result of flaws in the tissue collection protocol but related to the surgical approach, in which early ligation of perfusing vessels (in order to limit liberation of tumour cells into the circulation) may be responsible for a relative long peroperative warm ischemic interval. We contend that telomerase activity would be detected in almost if not all colorectal carcinomas

provided that the tissue samples are adequately preserved. If so, telomerase re-activation can be regarded as an obligatory step in colorectal carcinogenesis.

Telomerase activity was also studied in colorectal adenomas and in normal colorectal mucosa. Of the adenomas 47% (14/30) had detectable telomerase activity, whereas no such activity was found in histologically normal mucosa. The degree of dysplasia in the sample assayed for telomerase activity was highly significantly correlated ($P<0.0001$) with telomerase activation. All adenoma samples with HG dysplasia revealed telomerase activity, whereas only 20% of the adenomas samples with only LG dysplasia showed telomerase activity, generally at a lower level than in adenomas with HG dysplasia. Our results would indicate that telomerase activity is activated in adenoma at the stage of low grade dysplasia and present in all the areas with high grade dysplasia. They also suggest that telomerase positive LG dysplastic adenomas have a higher risk to progress towards HG dysplastic adenoma and colorectal carcinoma than do those without telomerase activity.

In the currently favoured model of colorectal carcinogenesis (16,32), APC mutations initiate the neoplastic process and the lesion progresses through accumulation of additional genetic alterations, implicating oncogenes and tumour suppressor genes (Fig. 2). K-*ras* mutations occur very early in 35%-45% of colorectal adenomas regardless of the degree of dysplasia and carcinomas (33,34). LOH of 18q, which might imply loss of function of DCC or DPC4, occurs in 50%-80% of HG adenomas, whereas *p53* mutations are limited in adenomas and occur in up to 70% of carcinomas (35,36). In our series, telomerase activation and K-*ras* mutation were not correlated; K-*ras* mutations occurred at a similar level in adenomas with or without HG dysplasia, which indicates that telomerase activation is a later event than K-*ras* mutation. Conversely, telomerase activity occurred with similar frequency in cancers and in adenomas with HG dysplasia. This would place telomerase activation before *p53* mutation in the molecular carcinogenetic concept of the adenoma-carcinoma sequence (Fig. 2).

In conclusion, we have demonstrated that under appropriately controlled conditions 100% of carcinomas and adenomas with HG dysplasia, but only 20% of LG dysplastic adenomas, show telomerase activity. Telomerase activation therefore

appears to be an essential step in the progression from a low grade to a high grade adenoma.

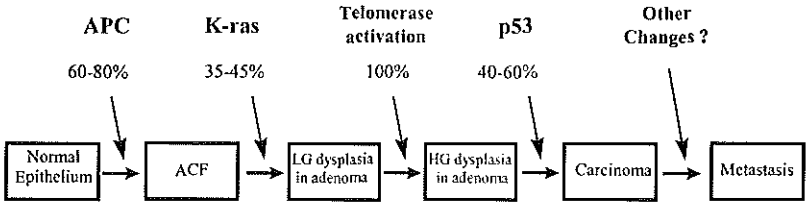

Figure 2: Genetic pathway of colorectal carcinogenesis. This model proposed by Fearon and Vogelstein is based on the concept of an adenoma-carcinoma sequence. All the molecular genetic alterations shown in this model rarely occur together in an individual. According to our results, telomerase activation will occurred between low grade to high grade dysplasia in adenomas with a very high frequency. ACF: aberrant crypt foci.

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CHAPTER IV

Expression of telomerase gene transcripts and correlation with telomerase activity in colorectal carcinogenesis

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SUMMARY

The human telomerase enzyme is composed of two essential components, hTR, which acts as a template for reverse transcription, and hTERT, which is the putative catalytic subunit for the enzyme. Recent studies have demonstrated a good correlation between hTERT expression and telomerase activation, whereas RT-PCR results seemed to reveal that hTR is ubiquitously expressed in all cells. These observations left unclear the role of hTR, and to a lesser extent hTERT, in the regulation of telomerase activation. In the present study, we examined the correlation of telomerase activity and the expression of these genes on a total of 70 colorectal tissues (25 adenocarcinomas, 30 adenomas and 15 normal colorectal mucosas). In our series, total RNA for RT-PCR analysis and cell extract for TRAP assay were obtained from consecutive sections and histological control was simultaneously performed. To avoid false-positive results, due to the fact that hTR cDNA and genomic hTR DNA are identical (the gene has no introns), extensive DNase digestion was performed before cDNA synthesis. RT-PCR analysis revealed hTERT mRNA to be expressed in all cancers and in 13 of 14 telomerase-positive adenomas, but never in telomerase-negative colorectal tissues. hTR transcripts were observed in all telomerase positive samples but also in 3 telomerase-negative samples, two adenomas and one normal colon mucosa. We conclude that hTERT and hTR expression are strongly correlated with telomerase activity. hTR transcripts, however, occur also in some telomerase negative tissues, and our results are in keeping with the concept that hTERT expression is a major regulator of telomerase activity.

Key words: Telomerase, Colorectal Carcinogenesis, RT-PCR, TRAP assay, hTERT, hTR.

INTRODUCTION

Telomerase, a ribonucleoprotein that catalyses the addition of TTAGGG repeats to the end of vertebrate chromosomes, is expressed in germ cells, in stem cells, in tumour cell lines and in the majority of tumours, but generally not in somatic cells (1-3). Telomerase reactivation might be a prerequisite for the development of malignant tumours from somatic cells. In humans, three major genes are responsible for the activation of telomerase: the human telomerase RNA (hTR), the telomerase associated protein (TP1) and the human telomerase reverse transcriptase protein subunit (hTERT). These telomerase component genes have been characterised recently (4-6).

TP1, of which the function as yet remains unknown, does not reflect the level of telomerase activity and is ubiquitously expressed (7,8). Thus, TP1 does apparently not play a crucial role during telomerase activation. It might be required *in vivo* for full telomerase assembly, function and regulation, but it can not be used as an indicator of telomerase activation.

hTR acts as a template for telomere elongation by the reverse transcriptase subunit. In an *in vitro* reconstitution experiment of human telomerase activity, hTR appeared to be an essential component (9). Tumour cells transfected with antisense hTR loose telomeric DNA, resulting in cellular senescence (4). These findings confirm that hTR is indispensable for telomerase activity. Whether or not hTR expression is an indicator of telomerase activity is a controversial issue. A correlation has been found between telomerase activity and hTR expression, as determined by in situ hybridisation (10-13). Recent studies, however, demonstrated that hTR RNA, as detected by RT-PCR, is widely expressed in both cancer and non-cancer tissues, although in cancer tissue at a higher level (7,8,10,14,15). The fact that the hTR gene has no introns requires specific consideration. hTR cDNA is identical to genomic hTR DNA and, as it is very difficult to avoid the presence of traces of genomic DNA in extracted RNA, during PCR amplification genomic hTR DNA might be co-amplified, leading to false-positive results. This might explain why, by RT-PCR, hTR RNA has been found in cancer and non-cancer tissues, irrespective of telomerase activity. This problem can be circumvented by extensive DNase digestion of the extracted RNA before cDNA synthesis. Very few RT-PCR experiments were performed with the use of DNase before cDNA synthesis (24-

27). In a recent report, normal urine samples were analysed and hTR was detected in only 15% of them whereas 83% of the urine samples from the patients with bladder cancer were found hTR-positive (24). In gastric tissues, Yasui et al have observed that telomerase activity was associated with increased hTERT and hTR expression (25). Unfortunately, in the other two papers, no normal tissues were analysed (26, 27).

hTERT has been identified as the putative human telomerase catalytic subunit (6). The expression of hTERT is closely correlated with telomerase activity *in vitro* and *in vivo* (6,16). Transfection of foreskin fibroblasts and retinal epithelial cells with plasmids encoding hTERT resulted in telomerase activity, telomere maintenance, and extension of life span (17,18). Regulation of the hTERT expression has been proposed as the major determinant of the regulation of telomerase activity. hTERT mRNA, as determined by either RNase protection or RT-PCR analysis, appears to be expressed at high levels in human tumours, cancer cell lines, and normal telomerase-positive tissues, but absent in telomerase-negative cell lines and in telomerase-negative tissues (6,15,16). Kolquist found hTERT mRNA by in situ hybridisation not only in carcinoma tissues but also in proliferative cells of normal tissues. Unfortunately, telomerase activity was not assayed in this study (19).

In a previous study, we have demonstrated that telomerase activation occurs in the progression from low grade to high grade adenoma (20). In the present study, the expression of hTERT mRNA and hTR RNA was determined by RT-PCR during colorectal carcinogenesis in order to determine which of these components might be primarily responsible for the regulation of telomerase activity.

MATERIAL AND METHODS

Tissue samples and TRAP assay

A total of 70 tissue samples were used in this study, including 25 colorectal carcinomas, 30 colorectal adenomas and 15 samples of normal colon mucosa. Adenomas were divided into two groups: adenomas with low grade (LG) dysplasia only; adenomas with at least focally high grade (HG) dysplasia. All tissue specimens were serially sectioned. The first and the last sections were cut at 5 μm and stained with hematoxylin and eosin for conventional microscopic examination by two independent pathologists. Telomerase activity and the presence of hTR RNA and hTERT mRNA transcripts were determined on protein and total RNA extract from the intermediate 12 μm sections. In a previous study, telomerase activity had been determined by the TRAP assay in the same samples (20). Briefly, depending on the size of the tissue sample, for each colorectal case 1 to 5 sections were sufficient to extract protein in 30 μl of CHAPS lysis buffer. Telomerase activity was determined using the TRAP assay according to manufacturer's protocol of the TRAPeze Telomerase Detection Kit (Oncor, Gaithersburg, MD, U.S.A.) and with three different concentrations of protein extracts: 0.1, 0.5 and 1 μg.

RNA preparation

Total RNA was extracted from tissue sections using Trizol (Life Technology) and, after ethanol precipitation in presence of 10 μg glycogen, resuspended in 20 μl of RNase-free water. About 0.6 to 1.0 μg of total RNA was subjected to 1% agarose gel electrophoresis. Good RNA quality was judged when 28S and/or 18S ribosomal RNA (rRNA) bands appeared on the agarose gel (21). Only the tissue samples with good RNA quality were used in our series.

To eliminate contaminating DNA, 5-10 μg of RNA was exposed to RNase-free DNase I (1 U/μg RNA, Roche Diagnostics, Germany) at 37°C for 30 minutes in 40 μl of a solution containing 40 mM Tris-HCL (pH7.9), 6mM $MgCl_2$, 10mM $CaCl_2$, 10mM NaCl, 10 mM DTT and 4U RNasine (Promega, Madison WI). The digestion was stopped by addition of 5 μl of a stop-solution (50mM EDTA and 1.5M Na-acetate).

The DNA-free RNA solution was re-extracted with phenol-chloroform and precipitated in ethanol in presence of 10 µg glycogen.

cDNA synthesis and PCR

cDNA was obtained from 2-5 µg of total RNA (treated or not with DNase) using Expand Reverse Transcriptase (Roche Diagnostics, Germany) and d(N)6 random primers as per manufacturer's instructions. For each sample, a parallel RNA was run with no Expand RT.

Two microliters (1/10) of the resulting cDNA was subjected to PCR, in a total volume of 20 µl, with the primers as follows: hTERT, 5'-TTCCTGCACTGGCTGATGAGTGT and 5'-CGCTCGGCCCTCTTTTCTCTG hTR, 5' -CGCCGTGCTTTTGCTCC and 5'-ACTCGCTCCGTTCCTCTTCC; p53 5'-TCTGGGCTTCTTGCATTCTGGGACA and 5'-TCTCGGAACATCTCGAAGCGCTCA; GAPDH 5'-GGGAAGGTGAAGGTCGGAGTC and 5'-AGCAGAGGGGGCAGAGATGAT. The amplification reactions were performed with an initial incubation step at 95°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, 65°C for 45 seconds, and 72°C for 45 seconds for hTERT; 35 cycles at 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds for hTR; and 30 cycles at 94°C for 30 seconds, 62°C for 45 seconds, and 72°C for 45 seconds for p53 and GAPDH, with a final incubation at 72°C for 10 minutes for all cDNAs. The reaction products were subjected to electrophoresis in 1.5 % agarose gel and visualised by ethidium bromide staining. The PCR procedure was performed at least twice for each sample.

RESULTS

RT-PCR analysis revealed the presence of hTERT mRNA in all 25 colorectal carcinomas, 9 of 10 (90%) adenomas with HG dysplasia and 4 of 20 (20%) adenomas with LG dysplasia (Table. 1 and Fig. 1). All but one of these samples were also telomerase positive. In only one case, an adenoma with HG dysplasia, the TRAP assay was positive but no hTERT mRNA could be detected by RT-PCR. No hTERT mRNA was found in 15 telomerase negative samples of normal colon mucosa. Therefore, expression of hTERT mRNA and telomerase activity coincided almost perfectly.

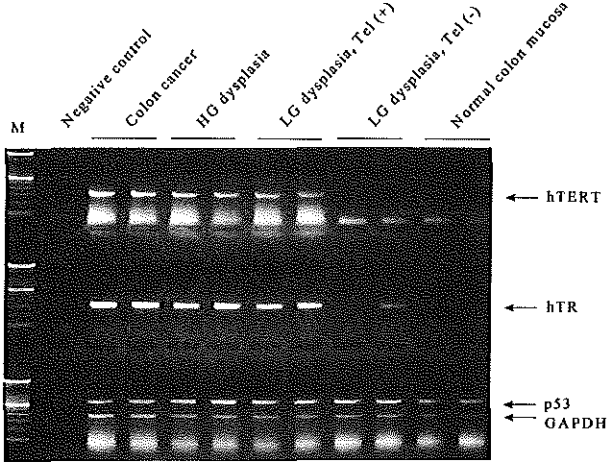

Figure 1: hTERT and hTR expression in human colorectal tissues determined by RT-PCR. Amplification of p53 and GAPDH were used to demonstrate the integrity of cDNA templates. Total RNA was treated with DNase I before cDNA synthesis. Results of telomerase activity, determined by the TRAP assay, are shown for each colorectal tissue. A size marker, 100 bp ladder, was also shown in lane M. In the negative control no RNA was added in the RT-PCR reaction.

Table I - Correlation between telomerase activity and expression of telomerase genes in colorectal tissues

	RNA expression / Telomerase positive	RNA expression / Telomerase negative	Number of cases
hTERT mRNA			
adenocarcinoma	25/25 (100%)	0	25
adenoma HG	9/10 (90%)	0	10
adenoma LG	4/4 (100%)	0/16	20
normal mucosa	0	0/15	15
hTR RNA			
adenocarcinoma	25/25 (100%)	0	25
adenoma HG	10/10 (100%)	0	10
adenoma LG	4/4 (100%)	2/16 (13%)	20
normal mucosa	0	1/15 (6%)	15

Correlation between telomerase activity and expression of telomerase subunits, hTERT and hTR, is statistically significant by chi-square test. ($P < 0.0001$)The PCR assay for hTR was positive in all tissue samples when RNA was not treated with DNase prior to PCR. Furthermore, an amplified band of the expected size was observed when the RNA was not reverse-transcribed (Fig. 2). These findings indicate that part of the amplified product might have come from DNA contaminating the RNA preparations. To eliminate this DNA contamination, the samples were treated with DNase I. The DNase-treated samples were investigated by PCR with and without prior reverse-transcription (RT), an amplified band of the expected size was never observed without RT (Fig. 2). By RT-PCR, hTR transcripts were present in all carcinomas, in all 10 adenomas with HG dysplasia, in 6 of 20 adenomas with LG dysplasia, and in only one out of 15 normal mucosa samples (Fig. 1). To test the possibility that DNase digestion might degrade RNA, undigested and digested RNA were subjected to

hTERT RT-PCR in these 70 colorectal tissues, and the same cases were found amplified. As shown in table 1, hTR specific transcripts were present in all colorectal tissues with telomerase activity, but also in two adenomas with LG dysplasia and one normal colorectal mucosa sample without telomerase activity ($P<0.0001$ by the chi-square test). The normal mucosa which gave a positive hTR result was histologically re-evaluated. The sample contained many lymphocytes, but it was also the case in four other normal mucosa samples without hTR RNA.

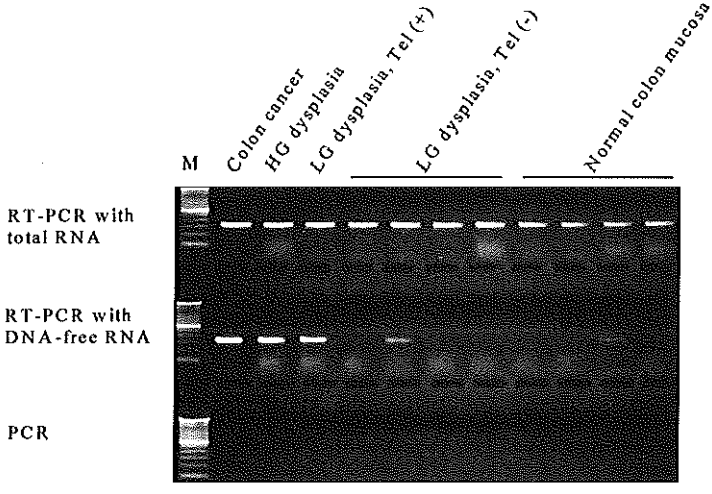

Figure 2: Expression of hTR determined by RT-PCR: influence of DNase treatment. Total RNA (without DNase treatment) or DNA-free RNA (with DNase treatment) samples were investigated with both PCR (when the RNA had not been reverse transcribed) and RT-PCR. Upper panel shows that hTR RNA expression was ubiquitously observed in cancerous and non-cancerous tissues, obviously due to the presence of genomic DNA in total RNA preparation (second panel). The third panel shows that hTR RNA is expressed at a high level in telomerase-positive tissues and at a low level in few telomerase-negative ones. Whereas no hTR product was obtained by PCR after DNase treatment (last panel). Results of telomerase activity, determined by the TRAP assay, are shown for each colorectal tissue.

DISCUSSION

To determine whether a close correlation exists between telomerase activity and expression of telomerase subunits, hTERT and hTR, during colorectal carcinogenesis, 70 colorectal tissues were investigated. For each sample, total RNA for detecting telomerase gene expression and protein for detecting telomerase activity were obtained from the same area of tissue samples with histological and tissue quality control.

Telomerase activity coincided almost perfectly with expression of both telomerase subunits, hTERT and hTR. hTERT was expressed in all except one telomerase-positive tissue sample, but never in telomerase-negative samples. A single discordant sample with telomerase activity and hTR transcripts but without hTERT mRNA was found. This might be explained by alternatively spliced hTERT transcripts, which might have been synthesised as observed in tissues during development (22,23), or by different rates of disintegration of the telomerase enzyme and the hTERT mRNA. As telomerase activity coincides almost perfectly with expression of the hTERT mRNA, this might be used as a marker for telomerase activity.

In an *in vitro* telomerase reconstituting experiment, it has been shown that hTR is an essential component of telomerase (9). However, to what extent hTR expression correlates with telomerase activation in human tissues is debated. Expression of hTR has been observed by RT-PCR in almost all the tissues analysed (7,8,10,14,15), whereas, by in situ hybridisation, hTR expression correlated well with telomerase activity (10-13). These contradictory results might be explained by a difference in sensitivity between RT-PCR and in situ hybridisation. If so, this implies that hTR might be expressed at least at a basal level in a wide variety of tissues. An alternative explanation might be an artefact of RT-PCR, i.e. amplification of genomic DNA sequences. RNA extracted from tissues is generally contaminated with a certain amount of genomic DNA. hTR DNA might be simultaneously amplified during hTR RNA amplification by RT-PCR, and thus lead to false-positive results. To test this hypothesis, we treated total RNA extracts with DNase to eliminate genomic DNA. With this additional step, hTR RNA was detected almost exclusively in telomerase-

positive cases. Nevertheless, hTR RNA was also found by RT-PCR in 3 telomerase-negative samples: two adenomas with only LG dysplasia and one normal colorectal mucosa. We conclude that previously reported hTR RNA transcripts as determined by RT-PCR in telomerase-negative samples are most likely due to amplification of genomic hTR DNA and not to a ubiquitous low rate of expression of the telomerase RNA component. In our series, only 10% (3/31) of telomerase-negative colorectal tissues expressed hTR. These results were in accordance with a recent report where DNase digestion was performed before cDNA synthesis and hTR RNA was detected in only 15% of normal urine samples but in 83% of urine from patients with bladder cancer (24).

In summary, our findings demonstrate that there is a very strong correlation between telomerase activity and the expression of hTERT mRNA and hTR RNA determined by RT-PCR in colorectal carcinogenesis. hTR transcripts, however, occur also in some telomerase negative tissues, and our results are in keeping with the concept that hTERT expression is a major regulator of telomerase activity.

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CHAPTER V

Telomerase activity and human telomerase reverse transcriptase (hTERT) mRNA expression in soft tissue tumors. Correlation with grade, histology, and proliferative activity.

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ABSTRACT

Telomerase activity (TA) is detected in most human cancers but, with few exceptions, not in normal somatic cells. Little is known about TA in soft tissue tumors. We have examined a series of benign and malignant soft tissue tumors for telomerase activity using the telomerase repeat amplification protocol (TRAP) assay. Analysis of the expression of the human telomerase reverse transcriptase (hTERT) was also carried out, using RT-PCR. TA was undetectable in benign lesions (15 of 15), low-grade sarcomas (6 of 6), and 50% (19 of 38) of intermediate-/high-grade sarcomas. Although the presence of TA in soft tissue tumors is synonymous with malignancy, it is neither a reliable method in making the distinction between reactive/benign and malignant (especially low-grade) lesions nor a reliable marker of tumor aggressiveness. Leiomyosarcomas and storiform/pleomorphic malignant fibrous histiocytomas rarely showed TA, irrespective of their grade. A strong correlation between hTERT mRNA expression and TA was observed, supporting the close relationship between both parameters. No significant relationship was observed between proliferative activity (as assessed by MIB-1 immunolabelling) and TA. We verified that the absence of telomerase expression was not due to the presence of telomerase inhibitors and therefore alternative mechanism(s) for cell immortalization, yet to be determined, seem to be involved in the development and/or maintenance of some soft tissue sarcomas.

INTRODUCTION

Telomerase is a complex enzyme containing both protein components and a RNA component. Its RNA subunit acts as a template for the synthesis of telomeric DNA, while a protein component, the human telomerase reverse transcriptase (hTERT/hEST2), catalyzes this process to make up for the inability of conventional DNA polymerase to replicate completely the ends of linear DNA (1, 2). Telomerase activity is detected in most human cancers as well as in some precancerous lesions and benign tumors (3-5). It is usually not detected in normal somatic cells except for normal human leukocytes (e.g. activated B and T lymphocytes), human germline tissues (adult testes and ovaries, but not mature spermatozoa and oocytes), and proliferating stem cells (5). Recently, based on a large number of samples, Meeker et al. (3) summarized the current data on telomerase activity in human cancers using the PCR-based TRAP assay. With the apparent exception of retinoblastoma which is often telomerase negative (6), the prevalence of telomerase activity in human cancers appeared to vary between 82% and 100% (3).

Owing to their rarity, ubiquitous location and significant morphologic diversity (more than 150 different histologic types described so far), soft tissue neoplasms are often a source of great difficulty in diagnostic pathology, especially when it comes to differentiating between benign and malignant lesions. Telomerase activity has been shown to be a potential useful diagnostic tool for the detection of cancer (5) as well as a potential prognostic marker for selected tumors (7-9). Apart from four sarcoma cases examined by Kim et al. in their study (10), telomerase activity has so far not been systematically examined in soft tissue tumors.

In the current study, we examined a series of benign and malignant soft tissue lesions for telomerase activity using the TRAP (Telomeric Repeat Amplification Protocol - TRAP) assay and for human telomerase reverse transcriptase (hTERT) mRNA expression. Since telomerase activation and human telomerase RNA (hTR) expression generally correlate with growth rate (11-13), we also examined sarcomas for proliferative activity using Mib-1 immunolabelling. We showed that reactive lesions, benign tumors, low-grade sarcomas and about 50% of intermediate-/high-

grade sarcomas were devoid of telomerase activity. When present, the latter correlated with hTERT expression but did not correlate with proliferative activity. We also showed that leiomyosarcomas and storiform-pleomorphic malignant fibrous histiocytomas are predominantly telomerase negative neoplasms.

MATERIALS AND METHODS

Tissue Samples. From 54 patients, 59 frozen tissue samples, including 15 benign soft tissue lesions and 44 soft tissue sarcomas, were retrieved from the tumor banks of the University Institute of Pathology of Lausanne, Switzerland and from that of the Department of Pathology of the Bergonié Institute, Bordeaux, France (JMC). Histologic typing and subtyping was performed on formalin-fixed, paraffin-embedded sections stained with hematoxylin and eosin, using the histological classification of soft tissue tumors of the World Health Organization (14). Additional techniques, including immunohistochemistry and electron microscopy, were used for diagnostic purposes if necessary. Histologic grade was established using the updated version of the French Federation of Cancer Centers grading system (15).

All tissue specimens were serially sectioned. Of 10 sections, the first and the last were cut at 5 µm and stained with hematoxylin and eosin for conventional microscopic examination, in order to assess tissue preservation and the relative amount of tumor tissue in the specimen available. Any specimen containing less than 30% tumor cells was excluded from the series. Intermediate sections were cut at 12 µm and put into two chilled 1.5 ml Eppendorf tubes, using a tapered glass pipette, for extraction of protein and total RNA. Depending on the size of tissue samples, between 1 and 5 tissue sections provided sufficient material for protein extraction in 30 µl of CHAPS lysis buffer for determination of telomerase activity. For a sufficient amount of RNA, 5 to 10 sections were necessary.

Total RNA was extracted and its quality assessed as previously described (16). Following extraction using Trizol (Life Technology) and ethanol precipitation in presence of 10µg glycogen, total RNA from soft tissue lesions was redissolved in 20µl of RNase-free water. About 0.6-1.0 µg of total RNA was subjected to 1% agarose gel electrophoresis. Preservation of 28S and/or 18S ribosomal RNAs species (rRNAs) were used to assess RNA degradation. Samples in which 28S and/or 18S RNA was no longer detectable were not tested for telomerase activity.

TRAP assay. Telomerase activity was determined using the TRAP assay with some modifications (17). SW480 colorectal carcinoma cells were used as a positive control in the PCR amplification. To this end, cell pellets (about 1×10^5 cells) were suspended in 400 µl of CHAPS lysis buffer. Frozen tissue sections (1 to 5 sections) were homogenized with 30 µl of CHAPS lysis buffer. After incubation for 30 min on ice, the lysate was centrifuged and the supernatant was immediately frozen at -80°C and stored until use. Protein concentration of the extract was measured by the BCA protein assay kit (Pierce Chemical Corp., Rockford, Illinois, USA).

In every case, the TRAP assay was performed using 3 different concentrations of the protein extract, 0.1, 0.5, and 1.5µg respectively. The protein aliquot was incubated with 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 100 µM dNTPs, 50 ng of TS primer (5'-AATCCGTCGAGCAGAGTT-3') in a thermocycler for 30 min at 30°C for the generation of telomeric repeats. After heating at 94°C for 2 min and cooling at 72°C, 1 unit of *Taq* DNA polymerase, 50 ng of ACX return primer (5'-GCGCGG[CTTACC]$_3$CTAACC-3'), 50 ng of NT internal control primer (5'-ATCGCTTCTCGGCCTTTT-3') and 0.01 amol of TSNT internal control (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'), were added to a total reaction volume of 25 µl. Then, 30 PCR cycles (94°C for 30 s, 56°C for 45 s and 72°C for 45 s) were performed. Five µl of PCR product was electrophoresed on a 8% polyacrylamide nondenaturing gel. The gel was stained with SYBR Gold (Molecular Probes, Eugene, Oregon, USA) and visualized under UV light using a charge coupled device (CCD) camera. The TRAP assay included the amplification of an internal control of 36 base pairs (bp); a false -negative result due to the presence of PCR/*Taq* DNA polymerase inhibitors was concluded when the 36-bp amplified product was not observed.

Analysis of hTERT expression by RT-PCR. Analysis of the expression of human telomerase reverse transcriptase (hTERT) was carried out by RT-PCR. cDNA was synthesized from 5-10 µg of total RNA using random primers. To amplify the reverse-transcribed cDNA, 2 µl aliquots of cDNA were subjected to 40 PCR cycles (95°C for 30 s, 65°C for 45 s and 72°C for 45 s) in 20 µl volume containing 10x Taq buffer, 50 ng of upstream primer 5'-TTCCTGCACTGGCTGATGAGTGT-3' and 50 ng of

downstream primer 5′ -CGCTCGGCCCTCTTTTCTCTG- 3′, 250 μM dNTPs and 1 U *Taq* DNA polymerase (Boehringer Mannheim). The primers correspond respectively to portion 1686 to 1708 and 1994 to 2014 of the published hTERT mRNA sequence (18). PCR products were analyzed on an 1.5% of agarose gel. The size of the hTERT PCR amplified product was 329 bp. The quality of cDNA was controlled by PCR amplification of p53 and GAPDH (glyceral-dehyde-3-phosphate dehydrogenase) transcripts.

Detection of telomerase inhibitors. Fourteen cases were examined for the potential presence of telomerase inhibitors. These included 4 sarcomas [Cases 18, 19, 23, and 24; Table 1] with undetectable telomerase activity despite hTERT mRNA expression and 10 lesions [Cases 16, 21, 22, 33, 42, 44, 49, 51, 54, and 58; Table 1] which were hTERT and telomerase negative. To this end, the TRAP assay was performed in parallel with cell extracts obtained from tumor tissue alone, tumor tissue mixed with 5 x 10^5 (5 μl) SW480 colorectal cancer cells, and SW480 cells alone. When only the first cell extract gave a negative TRAP assay (with the 36bp internal control amplified), this was taken to indicate the absence of telomerase inhibitors in the tumor tissue extract.

Immunohistochemistry. For all sarcoma cases, additional frozen sections were cut and stained with the Mib-1 antibody, recognizing the cell cycle-associated antigen Ki-67 (Immunotech SA, Marseille, France, diluted 1/10). Proliferation index was defined (after semiquantitative assessment of the staining) as low when less than 5%, moderate when 5 to 15%, and high when more than 15% of the nuclei were stained.

RESULTS

Fifteen reactive/benign lesions and 44 soft tissue sarcomas (STS) were included in our study. Of the latter, 6 tumors corresponded to local sarcoma recurrences [Cases 16, 18, 19, 24, 25, and 54] and 9 to sarcoma metastases located in the lungs [Cases 33, 34, 57, 58, and 59] and soft tissues [Cases 28, 30, 47, and 55] (see Table 1). From one patient, tissue samples from the primary tumor [Case 23] as well as from a locally recurrent lesion [Case 24] were available for examination. From another patient, the primary tumor [case 26] and two thoracic wall metastases [cases 30 and 47] and from a third patient a local recurrence [case 54] and a lung metastasis [case 33] could be analyzed. Of one patient we analyzed two lung metastases [Cases 57 and 58]. Two patients [Cases 23 and 53] received neoadjuvant chemotherapy prior to tumor sampling. The distribution according to histologic types and subtypes and histologic grade is shown in Table 1.

Telomerase activity was undetectable in benign soft tissue lesions (including 14 tumors and one myositis ossificans) whereas it was observed in 19 of 44 (43%) soft tissue sarcomas (STS). None of low-grade (grade 1), 9 of 16 (56%) intermediate-grade (grade 2), and 10 of 22 (45%) high-grade (grade 3) STS showed telomerase activity (Table 1; Fig. 1.). There was no correlation between histologic grade and telomerase activity using the Chi-square test (*p value = 0.06*). Only one [Case 52] out of 10 leiomyosarcomas and one [Case 59] out of 7 storiform/pleomorphic malignant fibrous histiocytomas expressed telomerase activity, irrespective of tumor grade. Most (5 of 6) locally recurring sarcomas failed to display telomerase activity. Five sarcoma metastases out of 9 (56%) were telomerase positive. Two sarcoma patients showed concordant telomerase activity status between the primary tumor and the corresponding recurrence [Cases 23 and 24] and between the primary tumor and the corresponding metastases [Cases 26, 30, 47].

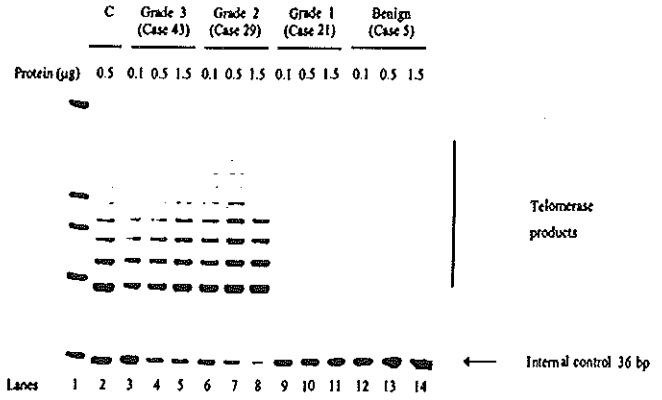

Fig. 1. Telomerase activity using TRAP assay in benign and malignant soft tissue tumors. Selected exemples of grade 3, grade 2, grade 1, and benign soft tissue tumors include an angiosarcoma (Case 43), a round cell liposarcoma (Case 29), an unclassified spindle cell sarcoma (Case 21), and an hemangioma (Case 5). The TRAP assay was performed using 3 different concentrations of the protein extract, 0.1, 0.5, and 1.5 µg respectively. Lane 1 : pGEM DNA size markers (Promega, Madison, WI, USA). Lane 2 : Colorectal cancer cell lines SW480 as a positive control.

hTERT mRNA was detected in 19 (44%) of 43 STS including 15 of 18 (83%; Case 26 not available) telomerase-positive and 4 of 26 (15%) telomerase-negative cases. A strong correlation between hTERT mRNA expression and telomerase activity was demonstrated using the Chi-square test (*p value < 0.0001*). None of the benign lesions expressed hTERT mRNA. In three cases (Cases 34, 38 and 41), marked telomerase activity was observed in the absence of detectable hTERT mRNA. A lack of expression of both enzyme activity and hTERT mRNA was observed in 21 of 43 sarcomas (49%; case 26 not available), recurrences and metastases included. Ten of these cases [Cases 16, 21, 22, 33, 42, 44, 49, 51, 54, and 58] were examined for the presence of telomerase inhibitors and all proved to be negative (Fig. 2.). In none of the 4 telomerase-negative, hTERT-positive sarcomas [Cases 18, 19, 23, and 24] was the lack of telomerase activity due to the presence of telomerase inhibitors.

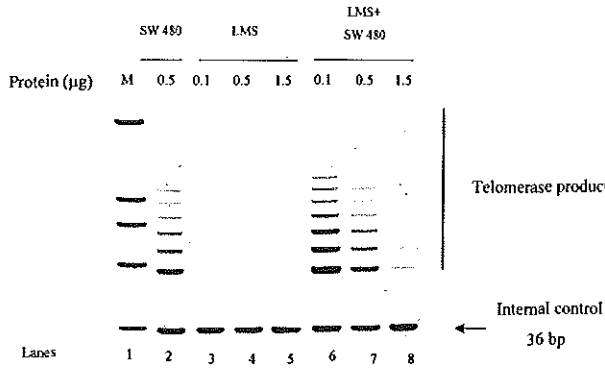

Fig. 2. Detection of telomerase inhibitors. Protein extracts from a leiomyosarcoma (LMS) (Case 51) were successively examined prior to and after complementation with extracts from SW480 colorectal cancer cells. The latter showed telomerase activity and was used as a control (lane 2). Telomerase activity was not detected in the protein extract from the leiomyosarcoma when taken in isolation (lanes 3, 4 and 5). After complementation with SW480, telomerase activity was restored (lanes 6, 7 and 8) indicating that the lack of telomerase expression in the leiomyosarcoma was not due to the presence of telomerase inhibitors. The amplified 36bp internal control acknowledges the absence of PCR/Taq DNA polymerase inhibitors. The TRAP assay was performed using 3 different concentrations of the protein extract, 0.1, 0.5, and 1.5 µg respectively. Lane 1 : pGEM DNA size markers (Promega, Madison, WI, USA). Lane 2 : Colorectal cancer cell lines SW480 as a positive control.

The MIB-1 (Ki-67) labelling index was assessed in 41 STS. Low and intermediate Mib-1 labelling indices were detected in 34 STS (83%). High labelling indices (>15%) were observed in the high-grade (G3) tumor category only. Local recurrences and sarcoma metastases showed Mib-1 labelling index equal or below 15% (see Table 1.). There was no relationship between Mib-1 indices and telomerase activity (*p value = 0.33*).

Table 1. Telomerase and hTERT mRNA expression in benign and malignant soft tissue tumors

Case no.	Histology	hTERT expression	TA	% Mib-1-positive nuclei
Benign lesions				
1	Lipoma	--	--	ND
2	Lipoma	--	--	ND
3	Intramuscular lipoma	--	--	ND
4	Intramuscular lipoma	--	--	ND
5	Intramuscular hemangioma	--	--	ND
6	Intramuscular hemangioma	--	--	ND
7	Hemangiomatosis	--	--	ND
8	Desmoid tumor	--	--	ND
9	Desmoid tumor	--	--	ND
10	Desmoid tumor	--	--	ND
11	Desmoid tumor	--	--	ND
12	Desmoid tumor	--	--	ND
13	Cellular schwannoma	--	--	ND
14	Ossifying myositis	--	--	ND
15	Localized giant cell tumor of tendon sheath	--	--	ND
Low-grade sarcomas (Grade 1)				
16	Well-diff. liposarcoma (R)	--	--	NA
17	Myxoid liposarcoma	--	--	1%
18	Myxoid liposarcoma (R)	+	--	1%
19	MFH, storiform-pleomorphic type (R)	+	--	10%
20	Leiomyosarcoma	--	--	NA
21	Unclassified spindle cell sarcoma / fibrosarcoma	--	--	1%
Intermediate-grade sarcomas (Grade 2)				
22	MFH, storiform-pleomorphic type	--	--	5%
23	MFH, storiform-pleomorphic type (post CT***)	+	--	1%
24	MFH, storiform-pleomorphic type (R)	+	--	1%
25	MFH, giant cell type (R)	+	+++	5%
26	Myxoid liposarcoma	NA	+	10%

27	Myxoid/round cell liposarcoma	+	++	2%
28	Myxoid + round cell liposarcoma (M)	+	+++	5%
29	Round cell liposarcoma	+	++	1%
30	Round cell liposarcoma (M)	+	+++	1%
31	Dedifferentiated liposarcoma	--	--	1%
32	Leiomyosarcoma	--	--	5%
33	Leiomyosarcoma (M)	--	--	1%
34	MPNST (M)	--	+++	1%
35	Myxoid chondrosarcoma	+	+	1%
36	Unclassified spindle cell sarcoma / fibrosarcoma	+	+	15%
37	Unclassified spindle cell sarcoma / fibrosarcoma	--	--	10%
High-grade sarcoma (Grade 3)				
38	DSRCT of the abdomen	--	+++	50%
39	MPNST	+	++	25%
40	MPNST***	+	+	70%
41	Embryonal rhabdomyosarcoma	--	+++	35%
42	Alveolar rhabdomyosarcoma	--	--	NA
43	Angiosarcoma	+	+++	70%
44	MFH, myxoid type	--	--	15%
45	Pleomorphic liposarcoma	--	--	5%
46	Pleomorphic liposarcoma	--	--	40%
47	Round cell liposarcoma (M)	+	++	10%
48	Synovial sarcoma (monophasic type)	+	+++	15%
49	Leiomyosarcoma	--	--	1%
50	Leiomyosarcoma	--	--	15%
51	Leiomyosarcoma	--	--	25%
52	Leiomyosarcoma	+	++	15%
53	Leiomyosarcoma (post CT***)	--	--	5%
54	Leiomyosarcoma (R)	--	--	1%
55	Leiomyosarcoma (M)	--	--	15%
56	Unclassified spindle cell sarcoma / fibrosarcoma	+	+++	2%
57	MFH, storiform-pleomorphic type (M)	--	--	10%
58	MFH, storiform-pleomorphic type (M)	--	--	10%
59	MFH, storiform-pleomorphic type (M)	+	+	1%

* - no activity; + low activity; ++ moderate activity; +++ marked activity

** % positive nuclei

*** Tumor grade assessed on incisional biopsy, prior to neoadjuvant chemotherapy.

MPNST: malignant peripheral nerve sheath tumor; MFH: malignant fibrous histiocytoma; Well-diff. liposarcoma: well-differentiated liposarcoma; DSRCT: desmoplastic small round cell tumor

(R): local recurrence; (M): metastasis

DISCUSSION

Telomerase activity has been shown to be activated in the majority of epithelial cancers (5) and may provide a useful diagnostic or prognostic indicator in epithelial but also glial neoplasms (7, 9, 11, 19). Little is known about telomerase activity and its potential diagnostic and prognostic implications in soft tissue lesions. The purpose of the currrent study was to clarify this issue.

Telomerase activity is undetectable in all benign lesions but also in all low-grade sarcomas as well as in approximately 50% of the intermediate-/high-grade sarcomas. This indicates that in all likelyhood a telomerase-positive tumor is a sarcoma, but when telomerase is negative, it has no value as a parameter for predicting behavior.

Before concluding, one must rule out the possibility of false-negative results. The TRAP assay used for the detection of telomerase activity is subject to limitations (16, 17, 20) and requires positive and negative controls. In the current study, every frozen tissue specimen was histologically controlled before submission to be sure that it was qualitatively and quantitatively representative of the tumor. Tissue quality is of vital importance for the success of the telomerase detection assay. Recently, we developed a quality test in which the preservation of 28S and/or 18S ribosomal RNAs species is used to assess total RNA degradation (16). Of the 99 mesenchymal lesions originally selected for this study, 59 only met the quality test requirements and were subsequently retained for analysis. A second reason for false-negative results might be the presence of *Taq* DNA polymerase inhibitors or telomerase inhibitors as illustrated in Hodgkin's disease. Until recently, Hodgkin's disease was thought to be a predominantly telomerase negative lesion (21). However, a recent study (22) showed that this apparent lack of telomerase activity was due to the presence of telomerase inhibitors, more specifically eosinophil-associated ribonucleases. The potential presence of inhibitors was carefully examined in the current study. To detect Taq DNA polymerase inhibitors, we used the TRAP assay described by Kim et al. (17) which includes the amplification of an internal control of 36 base-pairs (bp) in each assay, and a false-negative result is concluded when the 36-bp amplified product is not

observed. To detect telomerase inhibitors, protein extracts from each specimen were examined before and after spiking with telomerase-positive SW480 colorectal cancer cells. Absence of detectable telomerase activity in the spiked extract was taken to indicate the presence of telomerase inhibitors. No inhibitors could be detected using any of these methods.

A strong correlation between hTERT mRNA expression and telomerase activity was observed in 36 out of 43 (84%) STS ($p<0.0001$), hence supporting the close relationship between both parameters and the crucial role of the hTERT gene upregulation in telomerase activation (18, 23-26). Four hTERT-positive STS failed to express detectable enzyme activity and we showed that this was not due to the presence of telomerase/PCR inhibitors. The presence of alternately spliced hTERT transcripts deleted in critical regions of the reverse transcriptase (25, 26), abnormalities in the RNA template component of telomerase, or unbalanced levels of expression and/or posttranscriptionnal modifications of the different telomerase subunits (hTR, TLP1, and hTERT) (23, 26) could account for enzyme inactivity and, thus, explain those discrepancies. Telomerase activity was observed in the absence of detectable hTERT mRNA in 3 cases. In the latter situation, an increased degradation rate of the hTERT mRNA compared to that of the enzyme may explain this type of discordance.

A recurrent and crucial problem in diagnostic pathology is to differentiate true sarcomas from clinically benign but morphologically malignant-looking soft tissue lesions. Nodular fasciitis and myositis ossificans are prototypical exemples of such lesions. All benign lesions of our series (including a case of myositis ossificans) were negative for telomerase. Since 57% of sarcomas were also telomerase-negative lesions (including malignant fibrous histiocytomas and leiomyosarcomas), we therefore conclude that telomerase activity cannot be used to distinguish a benign lesion from a malignant one. This observation is of particular importance for the pathologist since malignant fibrous histiocytoma and leiomyosarcoma are those sarcomas which are most likely to be confused with pseudosarcomatous lesions. Along the same line, telomerase activity cannot be used to separate a well-differentiated fibrosarcoma from a desmoid tumor or a well-differentiated "lipoma-like" liposarcoma from a conventional lipoma, all four lesions being telomerase negative.

In about 50% of the intermediate- and high-grade STS, telomerase activity was undetectable. In addition, several locally recurring tumors and metastases did not express telomerase activity. This indicates that telomerase activity is not a reliable marker of aggressiveness in STS and cannot be used as a prognostic indicator in this tumor category, contrasting with what has been reported for epithelial neoplasms (9, 19), neuroblastoma (8), meningioma (7) and several other tumor types (5). Although our series included a limited number of cases per histologic category, which precludes definitive conclusions, telomerase activation might be histology related in being predominantly negative in leiomyosarcomas and malignant fibrous histiocytomas.

Neither telomerase activity nor hTERT mRNA was found in 21 of 43 (49%) STS, including two recurrences and four metastases. This suggests that telomerase activity is not an essential prerequisite for sarcoma development nor for its metastatic dissemination. Similar observations were made for transplantable osteosarcomas (27) as well as in renal cell carcinoma (28) and retinoblastoma (6). To explain the absence of telomerase activity in some tumors despite optimal tissue preservation and absence of telomerase inhibitors, the existence of an alternative telomerase-independent mechanism for cell immortality via telomere lengthening has been suggested (10, 29, 30). Although such a mechanism might be operative in some sarcomas and sarcoma cell lines (29, 30), it is unlikely to be universal. Indeed, a recent study (31) showed that about half of STS have short telomeres whereas in only 17% of the tumors examined the chromosomes had elongated telomere repeats. In addition, this study also showed differences in telomere length patterns between primary tumors and recurrences. Based on the latter findings, telomere lengthening, whether it occurs through telomerase activation or not, is unlikely to play a dominant role in sarcoma development and maintenance. Additional studies focusing on the relationship between telomere length and telomerase activity in STS are needed to clarify this issue. Another explanation which would account for the lack of telomerase activity in a significant number of STS is the fact that telomerase expression might be a field and/or a time dependent phenomenon. Indeed, intratumoral variations in telomerase expression have recently been documented in high grade astrocytomas (12) and it is conceivable that such a phenomenon occurs also in STS. Telomerase activity and human telomerase RNA (hTR) expression generally correlate with growth rate (11-13) and one might suppose

that the lack of telomerase activity in some STS is associated with low proliferative activity. Indeed, most of our telomerase-negative STS showed a low proliferation rate as assessed by Mib-1 staining suggesting a relationship between both parameters. However, this is hampered by the fact that 76.5% of telomerase-positive STS showed also low labelling indices (i.e. Mib-1 positive nuclei ratio equal or less than 15%), hence precluding any conclusions.

In conclusion, we showed that telomerase activity and hTERT mRNA are not expressed in benign mesenchymal lesions. In soft tissue sarcomas, telomerase activity is restricted to a subset of intermediate- and high-grade sarcomas, might be histology dependent and, as yet, cannot be used as a diagnostic or prognostic tool.

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CHAPTER VI

Detection of malignant effusions: comparison of a telomerase assay and cytologic examination

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ABSTRACT

Purpose: Telomerase activity is absent in most somatic cells, but it has been found to be reactivated in a majority of cancers. Our principal goal was to test whether the presence of telomerase activity concurred with positive cytology in detecting cancer cells in effusion diagnosis.

Patients and Methods: Telomeric repeat amplification protocol (TRAP) assay and cytological examination were performed in a blinded fashion on 91 pleural, peritoneal, and pericardial unselected fluids from 86 patients, without any change of routine cytologic sampling. In order to reduce the chance of a false-negative TRAP assay, the integrity of rRNA in the effusion cells was verified in each case.

Results: According to clinical context, 42% (38/91) of samples can be considered as malignant effusions by either cytologic examination of TRAP assay, whereas only 30% (27/91) were found malignant by cytology alone. Concordance between the telomerase assay and cytologic examination was observed in only 63 (69%) cases. Of the 28 non-concordant cases, 8 had a positive cytology without detectable telomerase activity. In 6 of these cases rRNA was almost completely degraded, and thus absence of telomerase activity might be due to bad cell preservation. Negative cytology in the presence of telomerase activity was found in 17 cases. Eleven of them were associated with a malignancy and thus a diagnosis of malignant effusion should be strongly suspected. For the 6 patients with telomerase activity in effusion and no preexisting cancer, a longer follow-up will be necessary.

Conclusion: These results suggest that telomerase activity is more often found in effusions than cytologically malignant cells. To some extent this indicates a higher sensitivity of telomerase assay for the detection of malignant cells than cytology, and thus the TRAP assay could become, in certain conditions, a useful adjunct in routine cytologic examination.

INTRODUCTION

Identifying tumor cells in body cavity effusions is a well-known diagnostic problem. Cytological examination is the most specific method for the diagnosis of a malignant effusion. Although the specificity of the test is very high, according to most studies the overall sensitivity ranges between 70% and 95% (1-3). Low sensitivity may be caused by presence of few malignant cells in the cell sample and by the limitations of the morphological approach: it can be difficult to differentiate between malignant and reactive cells. To improve the sensitivity of cytological examination, various approaches have been proposed, including immunocytochemistry (4,5), chromosome analysis (6), tissue culture techniques (7), DNA flow (8,9) or image cytometry (10), and cell image combined with immunocytochemistry (11). However, all these special techniques have a limited impact in routine cytological diagnosis. New approaches have to be developed in order to improve the sensitivity of cytological diagnosis in effusions.

The telomere-telomerase hypothesis of cell aging has rapidly become a focus of intensive research in the field of carcinogenesis. The hypothesis is based on the observation that telomerase activity is expressed in embryonic cells, in adult germline cells (12,13), and in most human tumors (12,14), but is undetectable in normal somatic tissues except for proliferative cells of renewable tissues including haemopoietic stem cells (15,16), basal cells of the epidermis (17) and intestinal crypt cells (18). Human telomeres, which are composed of many kilobases of TTAGGG repeats and associated proteins, protect and stabilize chromosome ends during DNA replication (19). In normal somatic cells telomeric DNA is reduced during each cell division. In contrast, tumor cells show a stability of average telomere length with cell division, suggesting that telomere stability may be required for cells to escape from replicative senescence, allowing them to proliferate indefinitely. Telomere length stability appears to be the consequence of telomerase activation. Telomerase, a ribonucleoprotein complex, is a specialized reverse transcriptase that synthesizes telomeric DNA sequences and thus restores telomere length (20,21). Telomerase activity has been detected in a great majority of human cancer tissues and cancer cell lines tested to date (22), and thus it may be an excellent marker to distinguish malignant cells from normal somatic cells.

Telomerase activity is generally detected by the telomeric repeat amplification protocol (TRAP) assay (12), which is a sensitive, reliable and reproducible test. Thus, testing for telomerase by the TRAP assay in body cavity fluids might be a approach to determine the presence of malignancy.

The aim of this study, therefore was to determine to what extent telomerase activity as measured by the TRAP assay could be an additional approach in routine cytological diagnosis to differentiate benign from malignant effusions.

PATIENTS AND METHODS

Patients and Cytological Samples. From November 1997 to April 1998, 91 unselected effusion samples were collected from 86 patients, submitted for cytological examination at the Department of Pathology of Lausanne. These included 49 men and 37 women aged 3 to 90 years (median: 63.9 years). The cytological material consisted of 59 pleural, 29 ascitic and 3 pericardial fluids. Telomerase assay and cytological examination were performed independently in a blinded manner. There was no exchange of information until the end of the study.

The fluid samples were used first for standard cytologic examination, including Romanowsky method and Papanicolaou stain. At the end of this examination the rest of the material was used for the telomerase assay. Cells in pleural, peritoneal or pericardial effusions were collected by centrifugation at 2'000 rpm for 10 min at 4°C. To avoid contamination with red blood cells that could potentially interfere with PCR, cells were washed 2 to 4 times with ice-cold hypotonic solution (50 mM KCl) and then kept frozen at -80°C.

Control of Tissue Quality. Cell integrity was measured using RNA integrity as a marker (23). Total RNA was extracted from 10-20 µl of frozen cells using Trizol (Life Technology), and after ethanol precipitation in presence of 10µg glycogen, the pellet was redissolved in 20 ml of RNase-free water. About 0.5 mg of total RNA was subjected to 1% agarose gel electrophoresis. If 28S and/or 18S ribosomal RNA bands appeared on the agarose gel, rRNA was at least partially intact. If no 28S and 18S rRNA bands presented, we concluded that rRNA was highly degraded.

TRAP assay. For tumor cell lines, around 10^6 cells were lyzed with 400 ml of CHAPS lysis buffer. For cytological materials, 10-20 ml of frozen cells were lyzed with 100 ml CHAPS lysis buffer. After incubation for 30 min on ice, the lysate was centrifuged and the supernatant was immediately stored at -80°C. Protein concentration of the extract was measured by the BCA protein assay kit (Pierce Chemical Corp., Rockford, Illinois, USA).

Telomerase activity was determined using the TRAP assay, according to the method reported by Kim and Wu (24), with some modifications. An aliquot of 0.5 mg of protein was incubated with 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 125 μM dNTPs, 50 ng of TS primer (5'-AATCCGTCGAGCAGAGTT-3') in a thermocycler for 30 min at 30°C for generation of telomeric repeats. After heating at 94°C for 2 min and cooling at 72°C, 1 unit of *Taq* DNA polymerase, 50 ng of ACX return primer (5'-GCGCGG[CTTACC],CTAACC-3'), 50 ng of NT internal control primer (5'-ATCGCTTCTCGGCCTTTT-3') and 0.01 amol of TSNT internal control (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'), were added to a total reaction volume of 25 ml. Then 30 PCR cycles (94°C for 30 s, 56°C for 45 s and 72°C for 45 s) were performed. Five ml of the PCR products were electrophoresed on a 8% polyacrylamide nondenaturing gels. The gel was stained with SYBR Gold (Molecular Probes, Eugene OR, USA) and visualized under UV light using a CCD camera.

Telomerase activity was also determined using a modified sensitive TRAP assay. Generation of telomeric repeats was performed as above but with 2.5 μg of protein. After extraction with phenol / chloroform, the DNA was precipitated with ethanol and air-dried. A 30 μl PCR mixture containing 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTPs, 100 ng of TS primer, 100 ng of ACX primer, 100 ng of NT internal control, 0.02 amol of TSNT internal control and 1 unit of *Taq* DNA polymerase, was added to the extracted DNA. Then 30 PCR cycles were performed as above.

RESULTS

Quality control of cytological materials.

For reliable TRAP assay results optimal preservation of RNA in the effusion cells under investigation is essential (23). We tested this by measuring integrity of ribosomal RNAs (rRNA). Only 53 of the 91 (58%) cytology samples had intact or only partially degraded rRNA; in the other 38 cases rRNA was highly degraded (Table 1 and Figure 1). The proportion of pleural and ascitic fluids with adequate rRNA preservation were very similar, 58 and 62% respectively. We subsequently examined the stability of rRNA in effusion cells left at 4°C, for prolonged periods of time. No systematic difference was observed between material after a short versus a long interval (Table 1).

Table 1: Stability of biological material in pleural, ascitic and pericardial fluids and relation to telomerase activity

	Total	Pleural	Peritoneal	Pericardial	Sampling <1 day	Sampling >1 day	TA +	TA-
Good RNA quality	53 (58%)	34 (58%)	18 (62%)	1 (33%%)	44 (59%)	9 (56%)	28 (53%)	25 (47%)
Bad RNA quality	38 (42%)	25 (42%)	11 (38%)	2 (67%)	31 (41%)	7 (44%)	8 (21%)	30 (79%)
Total cases	91	59 (65%)	29 (32%)	3 (3%)	75 (82%)	16 (18%)	36 (40%)	65 (60%)

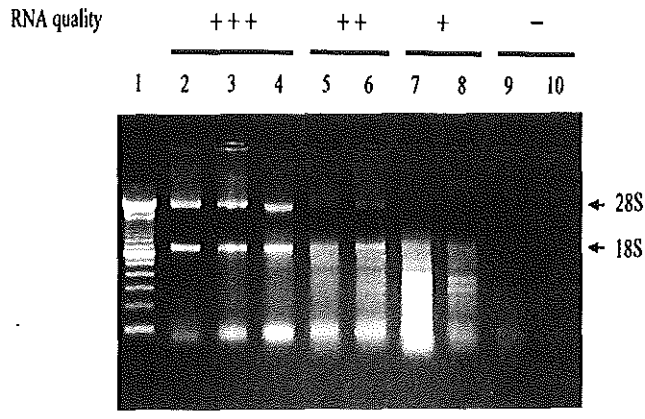

Figure 1: RNA quality in cytological effusions. Total RNA extracted from effusions (lanes 3-10) was subjected to 1% agarose gel electrophroresis. Lanes 2 to 10 contained about 0.5 µg of total RNA. Total RNA extract from the human colorectal carcinoma cell line SW480 was used as a positive control (lane 2). A size marker, the 100 bp DNA ladder (Life Technologies), was also shown in lane 1. 28S and 18S rRNA species were indicated by arrows.

Telomerase Activity in Body Cavity Fluids.

Telomerase activity was measured by both the standard TRAP assay described by Kim and Wu (24) and our modified sensitive TRAP assay (Figure 2). To obtain a higher sensitivity of the standard TRAP assay two approaches were tested. Increasing the number of PCR cycles led to a large number of false-positive results, probably due to artifacts in the reaction and the design of the PCR primers (data not shown). The assay was also performed with an increased amount of protein cell extract. This led to a strong inhibition of the PCR reaction. We solved this problem by introducing a phenol extraction and an ethanol precipitation step after the extension of the TS primer by the telomerase enzyme contained in the extracted protein sample. This permitted the use of larger amounts of protein cell extract without inhibition of the PCR reaction during the amplification step. Using this technique, we detected telomerase activity in 36 of 91 (40%) effusion samples. As expected, telomerase activity was more often ($p=0.002$) observed in effusions with relatively well preserved rRNA (28/53 cases, 54%) than in those with highly degraded rRNA (8/38 cases, 21%) (Table 1).

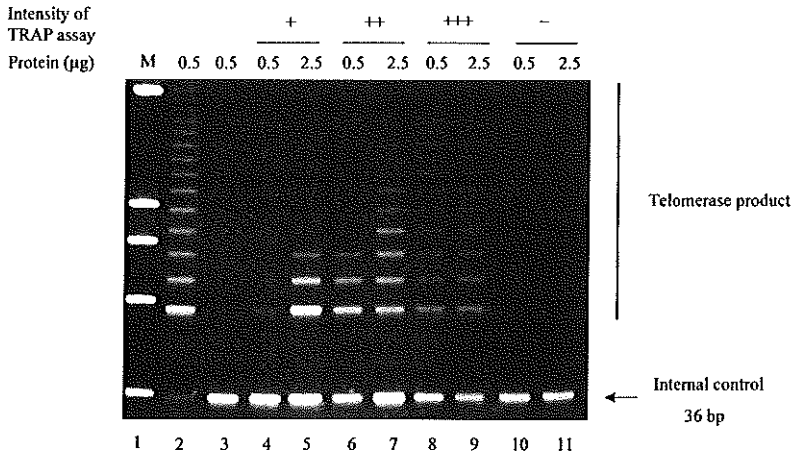

Figure 2: Telomerase activity in cytological effusions. From each effusion sample, telomerase assays were performed by the standard and by our modified sensitive TRAP assay using extracts containing respectively 0.5 and 2.5 μg of protein (lanes 4-11). The extract of the human colorectal carcinoma cell line SW480 was used as a positive control (lane 2). In negative control, no protein was added (lane 3). TRAP PCR products were indicated by a vertical line. The 36-bp internal positive control band was seen in every lane (indicated by an arrow), and it was used to identify non-informative specimens due to inhibitors of *Taq* polymerase. A size marker, pGEM DNA marker (Promega), was also shown in lane1.

Cytological Diagnostic and Telomerase Activity in Body Cavity Fluids.

The TRAP assay and cytological examination were performed in a blinded fashion on the effusion samples. All cases positive by the TRAP assay but were interpreted as negative by cytological examination were carefully rescreened by an experienced cytopathologist (R. B.). According to the cytological diagnosis achieved, effusions were classified into malignant (27 cases) and non-malignant (64 cases). In only 66 of 91 cases (73%) concordance between cytological examination and telomerase assay was observed; in 19 of 27 cases (70%) both were positive whereas 47 of 64 cases (73%) both were negative. The 28 (31%) non-concordant cases concerned pleural effusions (20 cases), ascitic (7 cases) or pericardial fluids (1 case).

Of the 27 cytologically malignant cases 8 (30%) were telomerase negative by the TRAP assay. In 6 cases poor rRNA quality was observed; false-negative telomerase activity in these samples may have been due to poor cell preservation

telomerase activity in these samples may have been due to poor cell preservation (Table 2, samples 1 to 6). The two other cases showed accurate malignant cytological diagnosis and the extracted rRNA was largely intact (Table 2, samples 7 and 8). The clinical evolution confirmed the cytological diagnosis and thus the TRAP assay failed to detect these 2 cases.

Of the 64 non-malignant effusions, 20 (31%) of them were found to be positive by the TRAP assay. Three cases were highly suspect by cytological examination, and tumor were finally identified by immunocytochemistry (Table 2, samples 9 to 11). In, 17 cytologically benign effusions telomerase activity was found. In 11 of these cases there was a strong clinical suspicion of malignant effusion (Table 2, samples 12 to 22). In 8 cases, the patient had an advanced malignant tumor proved histologically, which indicates that the sample assayed by telomerase very likely contained malignant cells (Table 2, samples 12 to 19). In 3 cases, curative treatment of a malignant tumor had been performed a certain time before the effusion developed. The presence of telomerase activity in the effusion might conceivably indicate re-appearance of malignant cells, even though cytological examination failed to identify them. In 6 effusions, a positive TRAP assay occurred in spite of a negative cytological examination, and without any suspicion of a malignancy (Table 2, samples 23 to 28).

In our series, several consecutive effusions were obtained from 4 patients. In two patients, both the cytologic examination and telomerase activity of effusions yielded a negative result. In the two others, the last obtained effusion gave a positive result for both techniques. Yet analysis of the previous effusions had shown positive results for the TRAP assay, but were negative for the cytologic examination.

Table 2: Discordant cases between cytological diagnosis and telomerase assay

Sample	Fluid	Inflammation.	RNA quality	Cytological diagnosis	TA	Follow-up	Conclusion
1	pleural	+++	–	+	–	adenocarcinoma	
2	pleural	+++	–	+	–	epidermoid carcinoma	*6 false-negative telomerase cases due to bad material*
3	pleural	+	–	+	–	B-cell lymphoma	
4	pericardial	–	–	+	–	breast carcinoma	
5	peritoneal	+	–	+	–	T-cell lymphoma	
6	peritoneal	++++	–	+	–	ovarian carcinoma	
7	pleural	++	+	+	–	epidermoid carcinoma	*2 true false-negative telomerase cases*
8	peritoneal	+++	++	+	–	adenocarcinoma	
9	pleural	+	+++	?	++	adenocarcinoma	*3 false-negative by cytology but + immuno **
10	pleural	+	+++	?	++	lung carcinoma	
11	peritoneal	++	+++	?	++	breast carcinoma	
12	peritoneal	+++	+++	–	++	breast carcinoma	
13	peritoneal	+	++	–	+	hepatocarcinoma	
14	pleural	++++	+++	–	+	pancreatic carcinoma	*8 true false-negative by cytology*
15	pleural	++++	–	–	+	liposarcoma	
16	pleural	+++	++	–	++	adenocarcinoma	
17	pleural	++++	–	–	+	lung carcinoma	
18	peritoneal	–	–	–	+	liver carcinoma	
19	pleural	+++	+++	–	++	adenocarcinoma	
20	pleural	+++	–	–	++	prostate adenocarcinoma	*3 potential false-negative by cytology*
21	pleural	+	–	–	+	colon adenocarcinoma	
22	pleural	+++	+	–	+	epidermoid carcinoma	
23	pleural	+++	+++	–	+	?	
24	pleural	+++	+	–	++	lupus	*6 potential false-positive telomerase cases*
25	pleural	+++	–	–	+	rib fractures	
26	pleural	+	–	–	++	rib fracture	
27	pleural	++++	++	–	+	post infection	
28	pleural	++++	–	–	+	BOOP	

+immumo: positivity by immunohistochemistry

DISCUSSION

Although analysis in cytology is presently considered the "gold standard", this approach has its limitations (1). Few pathological cells or pathological cells with relatively innocent morphology might escape attention. An important goal in the development of cytology would be the validation of new approaches that might efficiently supplement the conventional cytologic examination. Telomerase is expressed in almost all tumors and never in mature normal cells. Conceivably, the detection of telomerase activity could be a helpful tool to differentiate benign from malignant effusions. The results of our study indicate that the detection of telomerase activity by the TRAP assay might potentially constitute a useful tool for detecting cancer cells in effusions. Indeed, combining the two techniques of cytological evaluation and detection of telomerase activity, we were able to identify 42% (38/91) of effusions with a strong clinical suspicion of malignancy, whereas only 30% (27/91) of samples where found to be malignant by a unique cytological analysis.

Of 64 cytologically nonmalignant effusions, 17 were positive by the TRAP assay. In a majority of these cases (11/17, 65%), there was a strong clinical suspicion of malignancy, and thus telomerase detection using the TRAP assay was well correlated with the clinical status of the patients. Detection of cancer cells in effusions cytology may be influenced by the concentration and the morphology of cancer cells. The high sensitivity of the TRAP assay can ensure the detection of trace amounts of tumor cells in the presence of large excess of inflammatory or benign cells. In addition, some tumors do not show noticeable nuclear atypias and might therefore be interpreted as normal cells. These phenomena may lead to false-negative cytological diagnoses. However, this limitation does not affect the TRAP assay because this assay is independent of the morphology of the tumor cells.

For patients with telomerase positive effusions with presumably no preexisting cancer, additional studies are needed to understand better the factors associated with potential false-positive results. The role of large number of inflammatory cells in false-positive results has also to be considered (16). Telomerase activity was detected in 6 effusions from patients without evidence of cancer, and in 5 of them a large number of inflammatory cells was observed. However, the presence of inflammatory cells

(mainly lymphocytes) in our series, did not correlate with a positive TRAP assay. In 12 specimens with a large number of inflammatory cells and no or only partly degraded rRNA no telomerase activity was detected. Thus, presence of large amount of lymphocytes does not lead by itself to a telomerase positive assay. Among the 6 patients with no evidence of cancer and effusion positive for telomerase activity, two of them had a rib fracture. Telomerase activity observed in these pleural effusions was likely due to presence of some bone marrow cells, presumably hematopoietic stem cells (25,26), and not to cancer cells. Although the other 4 patients showed no evidence of cancer, we cannot exclude the possibility that an occult malignant tumor was present. Probably a long follow-up will be necessary to classify these cases as either accurate malignant effusions or true false-positive telomerase results. Recently, telomerase activity has been detected in the pancreatic juice of a patient 19 months before that a pancreatic cancer was diagnosed (27). In our series, two of the patients without evidence of malignant tumor had either a lupus erythematous or a Bronchiolitis Obliterans Organizing Pneumonia (BOOP), and some occult cancers have been shown to be associated with these diseases (28,29). Similar observation was done by other groups in some patients with tuberculosis (30,31), in this situation the appearance of a telomerase-positive pleural effusion could be due to undiagnosed concomitant cancer (32,33). Nevertheless, the occurrence of false-positive telomerase results due to cross-contamination between malignant and benign effusions also has to be considered. This is generally a main problem when PCR reactions have to be performed. We noticed that these 6 effusions were never in contact with an other telomerase positive case at the time of sample collection and preparation before freezing. However, this observation is not sufficient to exclude definitively any contamination with other malignant cases.

In some malignant effusions identified by cytologic examination, the TRAP assay failed to detect telomerase activity. One possible explanation may be that the cancer cells present in these effusions had no telomerase activity. To explain the absence of telomerase activity in some tumors the existence of an alternative telomerase-independent mechanism for cell immortality via telomere lengthening has been suggested (34,35). An other explanation could be due to the nature of the method used for measuring telomerase activity. Telomerase is a ribonucleoprotein enzyme that

uses its RNA as a template for the synthesis of TTAGGG repeats at the ends of the chromosomes. Thus, the feasibility of detecting telomerase activity by the sensitive TRAP assay is dependent of the quality of RNA and protein cell components. In a previous work, we observed that telomerase activity is not always detectable in cells with strongly degraded RNA (23). This could explain why telomerase activity was not observed in some malignant effusions. In our series, we observed that among the 8 malignant fluids were telomerase activity was not detected, 6 of them had highly degraded RNA. Furthermore, absence of telomerase activity was more significantly ($p=0.002$) observed in fluids with highly degraded rRNA (8/38 cases, 21%) than with relatively well preserved cells (28/53, 53%). Our results clearly indicate that for a valid TRAP assay, the control of rRNA quality is essential in order to reduce the number of false-negative results.

In conclusion, our results suggest that, provided that the cell sample is sufficiently well preserved to allow for undegraded rRNA, telomerase activity could be a very sensitive marker for the presence of malignant cells in effusions. In this study, telomerase activity was measured on routine sampling effusions, and thus this potential marker of malignancy could be used without any change of the standard cytological protocols. Nevertheless, before the telomerase assay becomes a useful adjunct in routine cytologic examination, two main questions have to be clearly settled. For patients with telomerase positive effusions with presumably no preexisting cancer, additional studies with a long-follow-up and an in situ hybridization analysis, probably coupled with an immunocytochemistry, will be needed to determine whether malignant cells are really present in these effusions. In routine diagnosis, due to the cost of this assay, it is not conceivable to test the presence of telomerase activity for all cytological benign effusions. Thus, a subgroup of them, with a relatively high percentage of telomerase positivity, has to be determined by a large scale prospective study. When these conditions will be achieved, the use of telomerase in combination with cytology will be more reliable in the diagnosis of malignant cells in effusions.

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CHAPTER VII

Concluding remarks and summary

7.1 Concluding remarks

Telomerase activity has been detected in almost all human cancer cell lines and in a majority of human cancers. Nevertheless, in about 10-15% of the reported human cancers telomerase activity was not detected (Kim et al, 1994). Several interpretations should be considered for these telomerase-negative tumour tissues. Firstly, evidence has been obtained that alternative mechanism(s) for lengthening of telomeres (ALT) might be involved in cellular immortalization, even though this remains elusive as yet (Reddel et al, 1997; Bryan et al, 1997). Secondly, assaying telomerase activity is subject to many pitfalls, including inadequate handing of samples, sampling artefacts and polymerase inhibitors. The positive internal control included in the TRAP assay covers the problem of *Taq* polymerase inhibitors. However, a tissue quality control has never been proposed to date. We detected, in a series of 50 colorectal carcinomas, telomerase activity in all of 25 cases with only partially degraded or intact RNA and in only 68% (17/25) cases with strongly or completely degraded RNA. This result demonstrates that in assaying telomerase activity tissue quality is of vital importance for reliable detection (Yan et al, 1998). Human cancers are heterogeneous in many ways, including in the pattern of genetic alterations (Saraga et al, 1997). In correlating histology with molecular parameters this should be taken into account in the experimental design. In a study of 30 colorectal adenomas, we observed close correlation between dysplasia grade and telomerase activity as determined on consecutive sections, which was lost when the grade established by routine pathological analysis was used. Clearly, proper controls and careful experimental designs are very important for getting more reliable results using TRAP assay. But, as pointed out by Meeker, many published studies have not made careful use of such control (Meeker and Coffey, 1997). In our studies, both tissue quality control and histological controls were included in all experiments.

As telomerase seems to be activated in some preinvasive neoplastic lesions, assaying telomerase activity might be used as an early diagnostic tool. At which moment in the multistep pathogenesis telomerase activated remains to be clarified for most types of cancer. The colorectal adenoma-carcinoma sequence is one of the best characterised models for multistep tumorigenesis. The gradual progression from adenoma to carcinoma allows detailed analysis of the morphological and molecular

stage at which telomerase is activated. Telomerase is activated in almost all colorectal carcinomas. Its presence in adenomas remained, however, somewhat controversial and no information was available in the relation between telomerase activity and grade of dysplasia in colorectal adenomas. We analysed telomerase activity, and in parallel with hTR RNA and hTERT mRNA subsequently in 25 colorectal carcinomas, 30 colorectal adenomas and 15 samples of normal colon mucosa. We found, strictly under controlled conditions, that all carcinomas and adenomas with high grade dysplasia, but only 20% of low grade dysplastic adenomas, show telomerase activity. These results indicate that telomerase is activated when adenomas progress from low grade dysplasia to high grade. This would place telomerase activation before p53 mutation but after K-ras mutation in Vogelstein's model of the molecular genetic pathway of colorectal carcinogenesis (Figure 1).

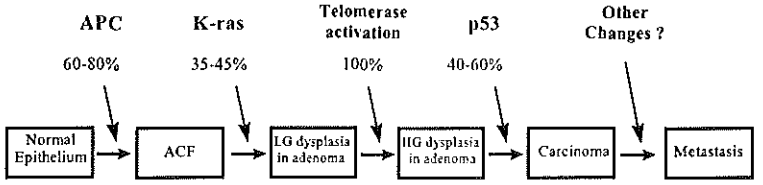

Figure 1. Genetic pathway of colorectal carcinogenesis. This model proposed by Fearon and Vogelstein is based on the concept of an adenoma-carcinoma sequence. All the molecular genetic alterations shown in this model rarely occur together in an individual. According to our results, telomerase activation will occur with a very high frequency between low grade to high grade dysplasia in adenomas. ACF: aberrant crypt foci.

hTERT and hTR have been identified as the major components of the enzyme in a reconstitution experiment (Weinrich et al, 1997). The expression of hTERT mRNA seems to be closely correlated with telomerase activity in cell lines and in human tissues, whereas hTR RNA as demonstrated by RT-PCR analysis is ubiquitously present in almost any tissue, irrespective of telomerase activity (Ramakrishnan et al, 1998; Takakura et al, 1998; Kyo et al, 1999). Consistent with other studies, we found that the expression of hTERT mRNA perfectly correlated with telomerase activation in our group of colorectal carcinomas. As no intron sequences

exist in the hTR gene, after the reverse transcription step, the sequence of hTR cDNA will be identical to that of genomic hTR DNA. To determine whether the ubiquitous expression of hTR might be due to a low level of transcription in various tissues or, alternatively, to amplification of genomic hTR DNA, extensive DNase I digestion before cDNA synthesis was performed in our study. After DNase digestion, hTR RNA was detected by RT-PCR mainly in telomerase-positive cases. Thus, when DNase digestion is omitted, the apparent broad expression of hTR RNA is probably due to the amplification of contaminating genomic hTR DNA and not to hTR expression. Our results indicate that telomerase RNA component (hTR) is not as widely expressed as previously thought.

Telomerase was found to be activated or upregulated in a majority of epithelial cancers and has been rapidly accepted as a potentially useful diagnostic and prognostic indicator in carcinoma and also more recently in gliomas. Little was known about telomerase activity and its potential diagnostic and prognostic use in soft tissue lesions. We examined a series of benign and malignant soft tissue lesions for telomerase activity, hTERT mRNA expression and Mib-1 labelling index. Telomerase activity was undetectable in benign soft tissue lesions and in low-grade soft tissue sarcomas, but detectable in 43% of the intermediate- and high-grade soft tissue sarcomas. A strong correlation between hTERT mRNA expression and telomerase activity was observed, but there was no correlation between Mib-1 labelling index and telomerase activity. The absence of telomerase activity was not due to the presence of telomerase inhibitors. An alternative mechanism for lengthening of telomeres (ALT) might be involved in the development and /or maintenance of some soft tissue sarcomas. Such a mechanism might be operative in some sarcomas and sarcoma cell lines, but it is unlikely to be universal. Recent data has shown that homogeneous distribution patterns of TRF (terminal restriction fragments) occurred in malignant schwannomas and malignant fibrous histocytoma. The former have shortened telomere length and the latter have normal telomere length. A very heterogeneous distribution pattern of TRF was perceived in liposarcomas (Schneider-Stock et al, 1998 and 1999). Therefore, additional studies in soft tissue sarcomas are needed to clarify the relationship between telomerase activity and telomere length. Our results indicate that telomerase activity is restricted to a subset of intermediate- and high-grade sarcomas

and, as yet, cannot be used as a diagnostic or prognostic tool. Sarcomas might provide an interesting model to study alternative mechanisms for lengthening of telomeres.

Several studies have shown that telomerase activity can be detected in almost any type of clinical specimen, including exfoliated cells and fine needle aspirates. Although cytological examination is presently considered to be the most specific method for diagnosis of malignant effusions, this approach has its limitations (Johnston et al, 1985). The detection of telomerase activity might be a helpful tool to differentiate benign from malignant effusions. In our study, 42% (38/91) of the effusions with a strong clinical suspicion of malignancy were positive by combining cytology and telomerase detection, whereas only 30% (27/91) of samples were positive by cytology only. Therefore, telomerase activity could be a very sensitive marker for the presence of malignant cells in effusions. Additional studies with long-follow-up and in situ analysis by in situ hybridization and immunocytochemistry are needed to determine whether malignant cells are really present in these effusions. Potentially, telomerase activation in combination with cytology might be more reliable than cytology only in the diagnosis of malignant cells in effusions.

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7.2 Summary

The telomere-telomerase hypothesis of cell senescence and immortalization has rapidly become a focus of intensive research in the field of carcinogenesis. In our studies, we investigated telomerase activity in colorectal carcinogenesis, in soft tissue lesions and in cytological specimens. **Chapter 2** describes the importance of tissue quality control in TRAP assay. Our results indicated that if cellular RNA is partially degraded or intact, more reliable results will be obtained. Contrary, if cellular RNA is strongly degraded, telomerase activity is not always observed. To avoid false-negative results due to sampling error, the control of RNA quality is an essential step in assaying telomerase activity. In **chapter 3**, with tissue quality control and histological control, we found that telomerase is activated in adenomas during progression from low grade to high grade dysplasia. We suppose that telomerase positive low grade dysplastic adenomas may have a higher risk to progress towards high grade dysplastic adenoma and carcinoma than do those without telomerase activity. Furthermore, we studied the expression of hTR RNA and hTERT mRNA in colorectal tissues in **chapter 4**. A significant correlation was observed between telomerase activation and the expression of hTERT mRNA and hTR RNA, determined by RT-PCR. As no intron sequences exist in the hTR gene, after the reverse transcription step the sequence of hTR cDNA will be identical to that of genomic hTR DNA. To avoid this problem, extensive DNase digestion was performed before hTR cDNA synthesis in our RT-PCR. In **chapter 5**, we examined telomerase activity in a series of benign and malignant soft tissue lesions and evaluated the relationship between telomerase activation and the expression of hTERT mRNA as well as Mib-1 labelling index. Telomerase activity was not observed in benign soft tissue lesions, in low-grade soft tissue sarcomas (STS), neither in about 50% of intermediate- and high-grade STS. A strong correlation between hTERT mRNA expression and telomerase activity was observed, but there was no relationship between Mib-1 labelling index and telomerase activity. Telomerase activity, as yet, can not be used as a diagnostic or prognostic tool in soft tissue sarcomas. Sarcomas might provide an interesting model to study the alternative mechanisms for lengthening of telomeres. In experiments of **chapter 6**, TRAP assay and cytological examinations were performed in 91 pleural, peritoneal and pericardial effusions. 42% (38/91) of the effusions with a strong clinical suspicion

of malignancy were positive by combining cytology and telomerase detection, whereas only 30% (27/91) of samples were positive by cytology only. Provided that sample of adequte RNA quality are used, telomerase activity could be come a helpful parameter for detecting malignant cells in effusion samples.

SAMENVATTING

In korte tijd heeft de telomeer-telomerase hypothese, ter veklaring van cellulaire veroudering en immortalisatie, belangrijke aandacht weten te trekken in het kanker onderzoek. In dit proefschrift wordt onderzoek beschreven naar telomerase activiteit in de colorectale carcinogenese, in weke delen tumoren en in cytologisch materiaal.

In hoofdstuk 2 wordt het belang van controle procedures voor de weefselkwaliteit bij de TRAP-assay besproken. Onze resultaten geven aan dat betrouwbare uitkomsten met deze test alleen dan verkregen kunnen wotrden als het cellulaire RNA intact of hooguit ten dele gedesintegreerd is. Als het RNA sterke desintegratie vertoont heeft een negatief testresultaat geen betekenis. Om fout-negatieve testuitslagen te voorkomen is het onderzoek naar de integriteit van het RNA derhalve onmisbaar.

In hoofdstuk 3 wordt onderzoek naar telomerase-activiteit in colorectale tumoren beschreven. Weefselkwaliteit werd bewaakt volgens de hierboven beschreven procedure en de monsters voor de TRAP-assay werden histologisch gecontroleerd op de aanwezigheid en klassificatie van tumor. Het bleek dat telomerase wordt geactiveerd bij de overgang van adenomen met laaggradige naar hooggradige dyslasie. We veronderstellen dat telomerase-positieve laaggradige adenomen een grotere kans hebben zich te ontwikkelen tot hooggradig adenoom of carcinoom dan telomerase negatieve.

Verder onderzochten we de expressie van hTR RNA en hTERT mRNA in colorectaal carcinoom. Dit onderzoek wordt beschreven in hoofdstuk 4. We vonden met behulp van RT-PCR een significante correlatie tussen telomerase activatie en de expressie van hTERT mRNA en hTR RNA. Omdat er in het hTR gen geen intronsekwenties voorkomen is het cDNA dat verkregen wordt na 'reverse' transcriptie van hTR RNA identiek aan de genomische hTR DNA sekwentie. Om co-amplificatie van genomisch DNA te voorkomen werd het monster behandeld met DNAse voorafgaand aan de hTR cDNA synthese.

In hoofdstuk 5 wordt onderzoek beschreven naar telomerase activiteit in een serie goed- en kwaadaardige weke delen tumoren. We evalueerden de correlatie tussen telomerase activiteit en de expressie van hTERT mRNA en de MIB-1 labeling index. Telomerase-activiteit werd niet gevonden in goedaardige lesies, evenmin in laaggradige weke delen sarcomen en in niet meer dan 50% van de intermediaire en hooggradige weke delen sarcomen. Er bleek een sterke correlatie te bestaan tussen telomerase activiteit en hTERT mRNA expressie maar niet met de MIB-1 labeling index. Telomerase activatie heeft derhalve bij de klassifikatie van weke delen tumoren geen praktische betekenis. Sarcomen zijn wel een interessant substraat voor onderzoek naar alternatieve mechanismen van telomeerverlenging.

In hoofdstuk 6 wordt onderzoek beschreven naar telomerase activiteit in cytologisch materiaal met behulp van de TRAP assay. Van 91 monsters van pleura-, pericard- en ascitesvocht met hoge klinische verdenking op maligniteit was 42% (38/91) positief in de TRAP assay en/of bij cytologisch onderzoek. Cytologisch onderzoek alleen was positief in slechts 30% (27/91) van de monsters. Voorop gesteld dat de RNA integriteit van het monster voldoende is, kan de bepaling van telomerase in een cytologisch monster wellicht een bijdrage leveren aan de detectie van tumorcellen in lichaamsvochten.

小结

目前,染色体末端和端粒酶假说已迅速成为肿瘤研究中的焦点。本研究侧重于端粒酶活性在结肠病变，软组织损伤及细胞学标本中的变化。

第一章. 综述端粒酶活性在肿瘤研究中的进展概况。

第二章. 重点描述了组织标本的质量在检测端粒酶活性方法(染色体末端重复扩增法)中的重要性。当从组织或细胞标本中提取的细胞 RNA保持其完整状态或仅部分被降解时，端粒酶活性检测的结果才是可靠的。反之，如果细胞RNA被完全降解，则很难检出端粒酶的活性。为了避免由于组织质量导致的假阴性结果，在端粒酶活性的检测中，RNA质量对照是个基本的条件。

第三章. 在严格的组织质量和组织形态学对照下，我们发现在结肠腺瘤中，端粒酶在低度发育不良到高度发育不良的过程中被激活。我们推测，与端粒酶阴性的低度发育不良腺瘤相比，端粒酶阳性的低度发育不良腺瘤可能具有更高的危险性进一步发展成高度发育不良腺瘤，甚至结肠癌。

第四章. 采用逆转录PCR的方法，我们在结肠组织(包括正常结肠组织，结肠腺瘤及结肠癌)中，检测了端粒酶 RNA基因(hTR)及端粒酶逆转录酶基因(hTERT)的表达。研究结果表明，端粒酶活性与hTERT mRNA及hTR RNA表达之间具有显著的关系。由于在hTR基因中缺乏内含子，所以在RNA逆转录成 cDNA 之后，hTR cDNA 和 hTR DNA在序列上是一样的。因此，在逆转录PCR反应中，hTR cDNA 和 hTR DNA可能同时被扩增而导致假阳性结果。为了克服这个问题，在hTR cDNA合成之前，必须用DNA酶消化RNA提取物中的基因组DNA。

第五章. 在一组良性软组织损伤和软组织肉瘤标本中，我们检测了端粒酶的活性，hTERT mRNA表达及细胞增长指数(Ki-67)。在15例良性软组织损伤及6例低度恶性软组织肉瘤中未检出端粒酶的活性。在得50%(19/38)的中度恶性及高度恶性软组织肉瘤中检出端粒酶的活性。我们发现在软组织肉瘤中，端粒酶的活性和hTERT mRNA表达之间的关系密切。但未发现端粒酶的活性和细胞增长指数之间具有密切关系。因此，端粒酶的活性的检测还不能用作软组织肉瘤的诊断和预后指标。但是，在维持染色体末端长度的替代机制研究中，软组织肉瘤大概可以作为一个模型。

第六章. 我们用染色体末端扩增法及细胞学检测法研究了91例胸腔，腹腔及心包渗出液标本。上述两种方法结合，可以检出42%(38/91)的临床高度可疑恶性肿瘤的病例。如果单靠细胞学方法，只能检出30%(27/91)。因此，我们认为端粒酶的活性可能在检测渗出液标本的恶性细胞中是个有用的指标。

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