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**Original Paper**

# Chondrosarcoma is not characterized by detectable telomerase activity

Judith V. M. G. Bovée[1]*, Lambert J. C. M. van den Broek[1], Anne-Marie Cleton-Jansen[1] and Pancras C. W. Hogendoorn[1]

*Department of Pathology, Leiden University Medical Center, P.O. Box 9600, L1-Q, 2300 RC Leiden, The Netherlands*

*Correspondence to:
Judith V. M. G. Bovée, MD, Department of Pathology, Leiden University Medical Center, P.O. Box 9600, L1-Q, 2300 RC Leiden, The Netherlands.
E-mail: J.V.M.G.Bovee@lumc.nl

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## Abstract

**Reactivation of telomerase, an enzyme which elongates human telomeres, is associated with cell immortilization. In approximately 90% of malignant tumours telomerase activity can be demonstrated, whereas in benign tumours it is mostly absent. Chondrosarcomas are relatively rare malignant cartilaginous neoplasms. A small number of chondrosarcomas located centrally in bone arise secondarily to an enchondroma, while the majority of chondrosarcomas developing from the surface arise within the cartilage cap of an osteochondroma. The histological distinction between a benign lesion and low-grade chondrosarcoma is generally considered difficult. To investigate whether the progression towards chondrosarcoma is characterized by reactivation of telomerase activity, this study determined telomerase activity in ten enchondromas, five osteochondromas, and 37 chondrosarcomas using the TRAP assay. In all tumour samples except one, telomerase activity was absent. By adding tumour lysates to the positive control, an increasing inhibition of telomerase activity was found with an increasing chondroid matrix, suggesting that it may contain inhibitory factors. Inhibition due to endogenous RNAse or *Taq*-polymerase inhibitors was excluded. The lack of detectable telomerase activity in the high-grade component of a dedifferentiated chondrosarcoma without matrix favours the possibility that telomerase is truly absent. Either its true absence or inhibitory effects disabling telomerase detection exclude the telomerase TRAP assay as a diagnostic tool in the differential diagnosis of benign and low-grade malignant cartilaginous tumours. Copyright © 2000 John Wiley & Sons, Ltd.**

**Keywords:** **telomerase; chondrosarcoma; enchondroma; osteochondroma; bone neoplasms**

## Introduction

Human telomeres are specialized structures constituting the ends of the eukaryotic chromosomes, consisting of TTAGGG repeats. Telomere integrity is important to maintain the stability of chromosomal ends, protecting chromosomes from recombination and loss. Telomeres shorten with each round of replication, since DNA polymerase cannot replicate the very end of a linear DNA molecule (the so-called 'end replication problem') [1,2]. Telomere loss has been suggested as a regulatory mechanism controlling the number of times a cell can divide before undergoing cellular senescence [1,2].

The maintenance of telomere length is associated with the re-expression of telomerase, a ribonucleoprotein with a reverse transcriptase activity that synthesizes TTAGGG telomeric repeat sequences, which are fused to the 3′ terminus of the chromosome. In immortal cell lines, germ cells (as in adult testis and ovaries), and proliferative cells of renewal tissues (such as activated lymphocytes), telomerase activity can be detected. Similarly, in malignant cells telomere length is maintained by telomerase, resulting in an immortal phenotype. Essentially all major types of cancer have been screened and the presence of telomerase activity has been detected in about 90% of cases [3]. In most somatic tissues and benign neoplasms, telomerase activity is absent [3]. A series of cartilaginous tumour samples has so far not been investigated.

Chondrosarcomas are malignant cartilage-forming tumours, presenting on average between 35 and 60 years of age. This is the second most frequent primary malignant bone tumour after osteosarcoma. Chondrosarcomas are slow-growing tumours characterized by the late development of metastases. It is considered difficult to assess the histological grade of cartilaginous tumours and to distinguish between benign tumours (enchondroma and osteochondroma) and those of low-grade malignancy [4]. The term 'borderline chondrosarcoma' was introduced for those cases where histological features are not sufficient to diagnose grade I chondrosarcoma, while X-rays show features of an aggressive neoplasm [5].

Only a small number of chondrosarcomas located centrally in bone (central chondrosarcomas) arise secondarily to an enchondroma, often in patients demonstrating non-hereditary multiple enchondromas (Ollier's disease or Maffucci's syndrome) [6]. The majority of chondrosarcomas developing from the surface of bone (peripheral chondrosarcoma) arise within the cartilage cap of a long-standing

osteochondroma (exostosis) [6,7], mostly in patients suffering from hereditary multiple exostoses (HME). HME is a familial disorder with an autosomal dominant mode of inheritance [8,9]. Malignant transformation is estimated to occur in 1–5% of cases.

So far, no diagnostic immunohistochemical or molecular genetic markers have been defined to help discriminate between benign and malignant tumours. We therefore determined telomerase activity in enchondromas, osteochondromas, and chondrosarcomas, including chondrosarcoma metastases, to investigate whether telomerase activity is reactivated during the progression from a benign cartilaginous precursor to its malignant counterpart.

## Materials and methods

### Clinico-pathological data

Fifty-two representative tissue samples of benign ($n=15$), borderline ($n=8$), and malignant ($n=29$) cartilaginous tumours were snap-frozen in 2-butanol on liquid carbon dioxide directly after surgery and stored at $-80^{\circ}$C. Tumour samples had been collected over the past 8 years. Chondrosarcomas developing centrally in bone (within the medullary cavity) as well as peripherally (from the cortex) were both included (Table 1). Examples are shown in Figure 1. Patient data were obtained by review of pathology specimens and reports, clinical charts, and radiographs. Central and peripheral chondrosarcomas were identified based on accepted clinico-pathological and radiological criteria [10]. Four central chondrosarcomas were secondary to an enchondroma [4], while all peripheral chondrosarcomas had developed within a pre-existing osteochondroma. Haematoxylin and eosin-stained cryostat sections were used to confirm the presence of viable cartilaginous tumour tissue. The amount of cartilaginous matrix was estimated as a percentage from the total surface area of the tissue slide and cellularity (including normal contaminating cells such as bone marrow) was semiquantitatively assessed by two observers independently.

In addition, we included two chondrosarcoma cell lines [SW-1353 (H5B-94, American Type Cell Cultures, Rockville, MD, USA) and OUMS-27 [11]], as well as a cell culture of a high-grade recurrent chondrosarcoma (99-389-1) [conditions as described [12], maintained in Hanks medium (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), fifth passage]. The cartilaginous nature of the cell lines was confirmed using S-100 immunocytochemistry and reverse transcription polymerase chain reaction (RT-PCR) for the cartilage-specific gene type II procollagen, as previously described [13]. Two metastases of central chondrosarcoma and both the low-grade cartilaginous and the high-grade anaplastic component (which often metastasizes) of a dedifferentiated chondrosarcoma were additionally included.

### Preparations of lysates

Up to 40 sections (20 μm) were cut from frozen tissue blocks of the cartilaginous tumour samples, normal costal cartilage, and testis. The cryostat sections were subsequently incubated with 200 μl of lysis buffer for 30 min on ice and centrifuged at 16 000 *g* for 20 min at 4°C. The supernatant was snap-frozen and stored at $-80^{\circ}$C. The protein concentration was determined using a Bradford assay (Pierce, Rockford, IL, USA). Lysates of cell lines were prepared using $2\times10^6$ cells, washed in phosphate-buffered saline (PBS), and centrifuged at 3000 *g* at 4°C. The pellet was suspended in 200 μl of lysis buffer. Lysates were frozen in aliquots and thawed only once.

### TRAP assay

The Telomerase PCR ELISA kit (Boehringer, Mannheim, Germany), a photometric enzyme immunoassay based on the telomeric repeat amplification protocol (TRAP) of Kim *et al*. [14], was used to detect telomerase activity. The assay was performed according to the manufacturer's instructions. In brief, two 10 μg protein aliquots were used to perform the telomerase assay in duplicate. Each aliquot was added to 25 μl of reaction mix, containing telomerase substrate, biotin-labelled P1-TS primer and P2 primer, nucleotides, and *Taq*-polymerase in Tris buffer in a final volume of 50 μl. PCR was carried out in a programmable heat block (Perkin Elmer, Norwalk, CT, USA) consisting of P1-TS primer elongation for 30 min at 25°C, telomerase inactivation for 5 min at 94°C, and product amplification by repeat of 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 45°C), and polymerization (90 s at 72°C). Five microlitres of the amplification product was incubated with 20 μl of the denaturation reagent (sodium hydroxide $<0.5\%$) for 10 min at room temperature; 225 μl of hybridization buffer (containing DIG-labelled detection probe) was added and briefly mixed. For each probe, 100 μl of this mixture was transferred into the wells of a streptavidin-coated microtitre plate, covered with the self-adhesive foil provided, and incubated for 2 h at 37°C. The hybridization mixture was removed and the wells were washed three times with 250 μl of washing buffer. Then the wells were incubated with 100 μl of anti-DIG-POD working solution (peroxidase-labelled sheep-anti-DIG to a final concentration of 10 mU/ml) for 30 min at room temperature. The wells were washed again five times and incubated with 100 μl of TMB substrate (3,3′,5,5′-tetramethylbenzidine) for 30 min at room temperature. The reaction was stopped with 100 μl of stop reagent ($<5\%$ sulphuric acid). Immediately afterwards, the absorbance was measured with a Titerskan Multiskan Plus at 450 nm. Telomerase activity was detected as absorbance at 450 nm greater than or equal to 0.250, according to the manufacturer's recommendations.

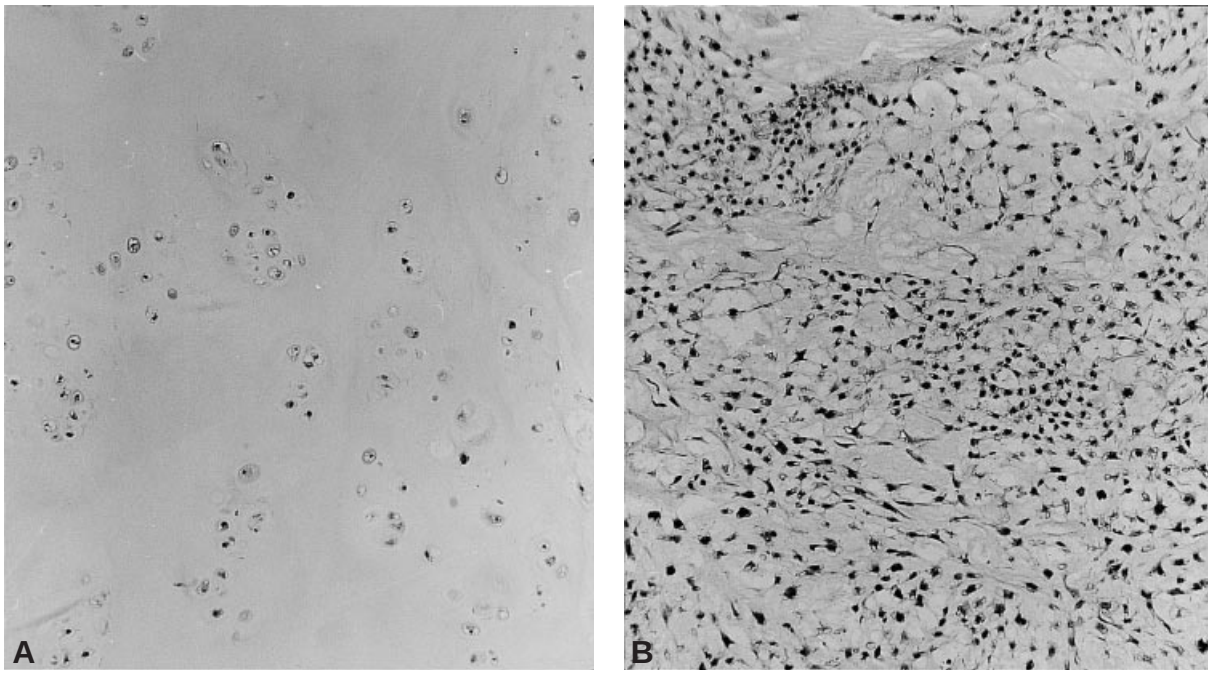


**Figure 1.** Histology of chondrosarcoma samples. (A) Representative slide of a resected specimen of a bulky grade I chondrosarcoma arising in the left proximal femur. The micrograph displays low cellularity with limited cytonuclear atypia and abundant chondroid matrix surrounding tumour cells. Scattered binucleated cells are found. (B) Light micrograph of a representative slide of a resected specimen from the right scapula showing a grade III chondrosarcoma. The micrograph displays high cellularity, cytonuclear atypia, and myxoid changes. Chondroid matrix is almost absent

The cell lysate provided by the manufacturer served as a positive control. In addition, 10 μg of testis lysate, prepared from tissue that was fresh frozen in the same manner as the chondrosarcoma tissue samples, was used to exclude the possibility that this process might inhibit the detection of telomerase. Negative controls included the addition of RNAse and were performed for each tumour sample.

## Results

### TRAP assay

Telomerase activity was not detected in any of the cartilaginous tumour samples under standard TRAP assay conditions, using cell lysates containing 10 μg protein (Table 1). One exception was weak telomerase activity in a single enchondroma ($A_{450}=0.305$). Absorbance values in the other tumour samples ranged from 0.052 to 0.214. Mean absorbance values per subgroup are shown in Table 1. Two chondrosarcoma cell lines (SW-1353 and OUMS-27) both demonstrated telomerase activity ($A_{450}=2.334$ and 0.871, respectively). A high-grade chondrosarcoma cell culture and the corresponding frozen tissue sample of the same tumour were both negative. Also, two metastases tested did not show any telomerase activity. Interestingly, in the dedifferentiated chondrosarcoma, both the high-grade anaplastic component and the low-grade cartilaginous component failed to show telomerase activity. Non-

Table 1. Mean absorbance values at 450 nm for each subgroup, divided into central and peripheral cartilaginous tumours

| Central tumours | Mean $A_{450}$ | Peripheral tumours | Mean $A_{450}$ |
|---|---|---|---|
| Enchondromas ($n=10$) | 0.115 | Osteochondroma (n=5) | 0.079 |
| Borderline CS ($n=7$) | 0.085 | Borderline CS ($n=1$) | 0.087 |
| Primary CS I ($n=6$) | 0.076 | Secondary CS I ($n=4$) | 0.082 |
| Primary CS II ($n=11$) | 0.084 | | |
| Primary CS III ($n=3$) | 0.065 | | |
| Secondary CS I ($n=4$) | 0.081 | | |
| Dedifferentiated CS ($n=1$) | | | |
| Cartilaginous component | 0.073 | | |
| Anaplastic component | 0.069 | Controls | Range |
| Metastases ($n=2$) | 0.087 | Positive controls | 0.835–2.508 |
| Normal cartilage | 0.070 | Negative controls | 0.048–0.193 |

CS = chondrosarcoma, I, II, III: tumour grades according to Evans [29]. All tumour samples were negative, since telomerase activity was detected as absorbance at 450 nm greater than or equal to 0.250. Positive controls included the cell lysate provided by the manufacturer and testis lysate prepared from fresh frozen tissue.

neoplastic costal cartilage failed to show any telomerase activity, as expected ($A_{450} = 0.070$). In addition, one chondrosarcoma stored at $-80°C$ for only 8 days was subjected to the telomerase assay. This sample also failed to show telomerase activity, excluding the lack of telomerase activity resulting from longer storage of tissue blocks at $-80°C$.

Both the cell lysate provided as a positive control by the manufacturer and the testis lysate demonstrated high telomerase activity each time the assay was performed ($A_{450}$ range 0.835–2.508).

### Inhibition tests

Lack of detectable telomerase activity in chondrosarcoma due to lack of sensitivity of the TRAP assay was excluded, since the lysis buffer according to Norton *et al.* [15], which would yield higher amounts of telomerase, did not result in detectable telomerase in three tumour samples. For six tumour samples, the assay was carried out with a 25-fold increased protein concentration (250 μg instead of 10 μg), which did not result in detectable telomerase activity.

Addition of the RNAse inhibitor RNAsin (1 U/μl) (Promega, Madison, WI, USA) to the lysis buffer prior to cell lysis [16,17] did not restore telomerase activity in three chondrosarcoma samples, excluding the possibility that tumour cells contained high levels of endogenous RNAse, resulting in inactivation of telomerase upon lysis. Two phenol extractions and an ethanol precipitation prior to the PCR to remove possible *Taq* inhibitors [16] did not restore telomerase activity in three chondrosarcoma samples, suggesting that lack of detectable telomerase due to *Taq*-polymerase inhibitors is less likely.

In order to determine whether the cartilaginous tumour lysates could inhibit telomerase activity of the positive control, lysates (10 μg) of 15 tumours [enchondroma ($n = 2$), chondrosarcoma borderline ($n = 3$), grade I ($n = 2$), grade II ($n = 4$), grade III ($n = 3$), and the anaplastic component of the dedifferentiated chondrosarcoma] and normal cartilage were added to 10 μg of testis lysate. Interestingly, a trend was seen in which the addition of lysate of normal cartilage and of tumour samples with a high matrix percentage and low cellularity (as is usually found in benign and low-grade tumours) resulted in strong inhibition of telomerase activity. Conversely, tumour samples with a low matrix percentage and high cellularity gave less strong inhibition of telomerase activity (Figure 2). Surprisingly, although the anaplastic component of dedifferentiated chondrosarcoma, which has high cellularity and no chondroid matrix, did not show detectable telomerase activity, only slight inhibition was found in the inhibition assay. The addition of bovine serum albumin (BSA, grade V) or lysate buffer did not result in inhibition of telomerase activity.

## Discussion

The development of peripheral chondrosarcoma within a pre-existing osteochondroma results in genetic instability, as we previously demonstrated by a high percentage of loss of heterozygosity (LOH) and a wide variation in DNA ploidy in peripheral chondrosarcoma [18]. In contrast, peridiploidy and a low percentage of LOH in central tumours suggested that a different oncogenic molecular mechanism, possibly consisting of a single genetic alteration, may be operative [18]. Both of the benign precursor lesions, enchondroma and osteochondroma, have been shown to be clonal and to show little or no growth [19,20]. Unfortunately, no specific immunohistochemical or molecular genetic markers that characterize the progression from enchondroma to central chondrosarcoma or osteochondroma and to peripheral chondrosarcoma have been found so far to assist in the differential diagnosis. It is generally considered difficult to distinguish between benign cartilaginous tumours and low-grade chondrosarcoma [4]. Since all major types of cancer demonstrate telomerase activity in the vast majority of cases, whereas telomerase activity is detected in a much lower percentage of benign lesions [3], we postulated that reactivation of telomerase activity would accompany malignant transformation in cartilaginous tumours.

Surprisingly, telomerase activity was not detected in any of the 52 cartilaginous tumour samples using a standard TRAP assay, apart from weak activity in a single enchondroma. Chondrosarcomas generally display low cellularity, especially in low-grade tumours, with a low proliferative index [18]. For other tumours, it was suggested that highly proliferative tumours display a higher telomerase activity [21]. It is, however, unlikely that this explains the complete lack of telomerase activity, since the TRAP assay is highly sensitive, detecting telomerase activity if as few as 1 in $10^4$ cells in a mixed population has telomerase expression [14]. We confirmed the presence of viable tumour cells by investigation of haematoxylin and eosin-stained cryostat sections. Increasing the protein concentration 25-fold did not result in detectable telomerase activity. In addition, the use of a lysis buffer prepared according to Norton *et al.* [15], which would yield higher amounts of telomerase, failed to demonstrate telomerase in all chondrosarcoma samples tested.

Other tumour types with absent or infrequent telomerase activity have been reported. Lack of telomerase activity has been described in 50% of retinoblastomas [22], which was explained by the fact that retinoblastoma is of embryonic origin and develops at an early age, when telomeres are still relatively long [22]. In contrast, chondrosarcoma is a tumour of late adulthood (on average between 35 and 60 years of age). In addition, 21 of 21 desmoids [23], 25% of glioblastomas [24], and nine of ten leiomyosarcomas [25] were reported to be telomerase-negative. In six

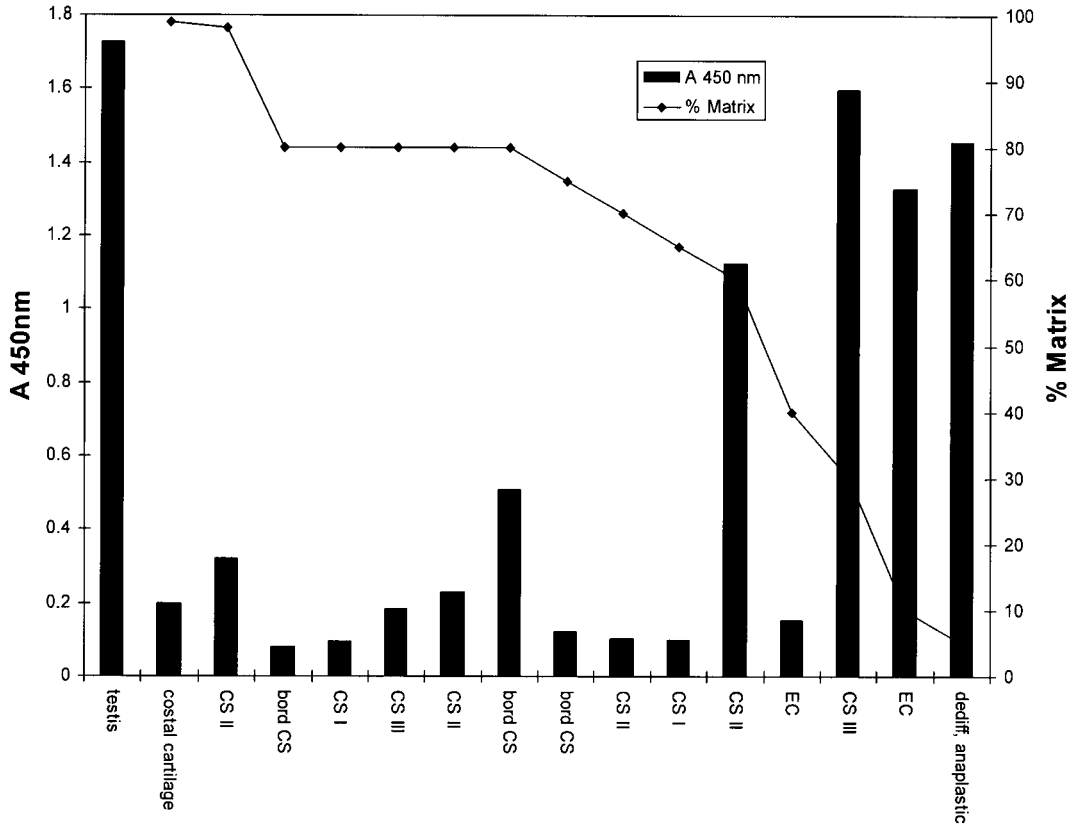


**Figure 2.** Results of the inhibition assay. On the left axis, the absorbance at 450 nm is demonstrated, represented in bars, whereas on the right axis, the amount of cartilaginous matrix is demonstrated, estimated as a percentage from the total surface area of the tissue slide. Due to admixture with normal tissue, ranking of the tumours according to the percentage of hyaline matrix is not according to histological grade. The left bar represents the positive control of the testis lysate, to which the cartilaginous tumour lysates were added

low-grade soft tissue sarcomas telomerase activity was absent, while only 19 of 38 (50%) intermediate/high-grade soft tissue sarcomas demonstrated telomerase activity [25]. Also, 10–30% of immortal cell lines lack telomerase expression [16,26,27]. This phenomenon may be explained by a novel and as yet unidentified alternative pathway (denoted alternative lengthening of telomeres, ALT) to maintain telomere length [16,26,27].

There are two possible explanations for the lack of detectable telomerase activity in chondrosarcoma. Either the detection of telomerase is just strongly inhibited in cartilaginous tumour lysates, or telomerase activity is truly absent in chondrosarcoma. Eosinophil or neutrophil granulocytic infiltrates, described as inhibiting telomerase detection in Hodgkin's disease due to the presence of RNAses, are characteristically absent in chondrosarcomas [17]. Inhibition due to another endogenous RNAse was excluded as well, using the addition of an RNAse inhibitor upon cell lysis. Inhibition of *Taq*-polymerase was not very likely, since phenol extractions and an ethanol precipitation to remove possible *Taq* inhibitors did not result in the detection of telomerase. We did, however, demonstrate the inhibition of detectable telomerase activity of testis lysate by adding cartilaginous tumour tissue lysates. A trend was seen for the addition of lysate of normal cartilage and of tumour samples with a high matrix percentage and low cellularity (as is usually found in benign and low-grade tumours) to result in strong inhibition of telomerase activity. Conversely, tumour samples with a low matrix percentage and high cellularity gave less strong inhibition of telomerase activity (Figure 2). Our results may therefore suggest that cartilaginous tissue, especially the hyaline cartilaginous matrix, may contain unusually strong inhibitory factors other than endogenous RNAse or *Taq*-polymerase inhibitors, disabling the detection of telomerase activity using the TRAP assay.

However, we tested two chondrosarcoma cell lines, OUMS-27 and SW-1353, and both demonstrated telomerase activity. Chondrosarcoma cell lines are sparse, since chondrocytes are difficult to culture *in vitro*, because of their limited proliferation potential. They usually cease dividing after several generations. We succeeded in culturing a recurrence of a high-grade peripheral chondrosarcoma, with almost no chondroid matrix that had been passaged five times before testing, which was therefore not yet an established cell line. No telomerase activity could be detected. Our results therefore suggest that additional aberrations *in vitro* reactivating telomerase would be necessary, in a late

passage during cell culture, to establish a chondrosarcoma cell line. This may also explain why it is difficult to establish a chondrosarcoma cell line with a cartilaginous phenotype. Furthermore, the absence of telomerase in the early passages during high-grade chondrosarcoma cell culture suggests that telomerase activity is truly absent in chondrosarcoma.

Surprisingly, although only slight inhibition of detectable telomerase activity of the testis lysate was found in the inhibition assay using the anaplastic component of dedifferentiated chondrosarcoma, no telomerase activity could be detected. Dedifferentiated chondrosarcoma consists of a mostly low-grade cartilaginous and a high-grade anaplastic component, of which both have been shown to be derived from a single precursor [28]. The anaplastic component is very cellular, contains no cartilaginous matrix, and has a very high metastatic potential. The complete absence of detectable telomerase activity in the anaplastic component of dedifferentiated chondrosarcoma, as well as in high-grade conventional chondrosarcomas, largely devoid of any cartilaginous matrix, may additionally favour the possibility that telomerase activity is truly absent in chondrosarcoma.

In conclusion, we have shown the *in vitro* inhibitory effect of a tissue component, probably the cartilaginous matrix, on telomerase activity detection using the TRAP assay. We have demonstrated the absence of detectable telomerase activity in benign as well as malignant cartilaginous tumours, even those with little or no cartilaginous matrix. However, the lack of detectable telomerase activity in the high-grade component of a dedifferentiated chondrosarcoma without matrix, and in a high-grade chondrosarcoma cell culture, favours the possibility that telomerase is truly absent. Due either to the absence of telomerase in chondrosarcomas, or to inhibitory effects disabling telomerase detection, the telomerase TRAP assay is excluded as a diagnostic tool in the differential diagnosis of benign and low-grade malignant cartilaginous tumours.

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