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Telomere length and expression in astrocytic brain tumors

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Zusammenfassung

Telomere befinden sich an den Enden der linearen Chromosomen um das Genom zu schützen und zu stabilisieren. Ein Telomere besteht aus TTAGGG-Wiederholungen von DNA Sequenzen. Erst kürzlich wurde gezeigt, dass Telomere in TERRA (Telomere repeat containing RNA) umgeschrieben werden. Diese nicht-kodierenden RNAs spielen eine Rolle für Telomere erhaltenden Mechanismen (TMM) und für die Organisation von Chromatin. Die Transkription von TERRA startet im Bereich der Subtelomere und hängt vom epigenetischen Zustand der Telomere ab. Astrozytome verwenden zwei TMM, einerseits Telomeraseaktivität (TA) und andererseits einen alternativen Mechanismus (ALT = alternative lengthening of telomeres).Der ALT-Mechanismus führt in der Regel zu längeren Telomeren als der TA-Mechanismus. Eine Korrelation zwischen TMM und dem Überleben von Patienten wurde für hochgradige Astrozytome gefunden, wie für Glioblastoma multiforme (GBM). Diese Erkenntnis und die schlechte Prognose für Patienten mit GBM führen zur Notwendigkeit der weiteren Charakterisierung von TERRA in diesen Tumoren.

In dieser Studie wurden Tumor-Gewebeproben (ts) von 46 Patienten mit Diagnosen Astrozytome WHO-Grad II bis IV untersucht: 12 diffuse Astrozytome (DA) Grad II, 6 anaplastische Astrozytome (AA) Grad III und 28 GBM Grad IV. Ergänzend wurden Tumor-Zelllinien untersucht, 12 etabliert aus Astrozytome und eine ALT-Zelllinie aus einem primären Osteosarkom. RNA und DNA wurden isoliert. Um die Relative Menge (RQ) der Transkriptionslevels zu messen, wurde die RNA in DNA umgeschrieben und eine Real Time PCR durchgeführt. Expressionslevel wurden mit jenen der house-keeping Gene Beta-Aktin und 36B4 verglichen. Die Telomerlänge wurde mit TRF Analyse und Real-time PCR bestimmt. Relative Mengen wurden zwischen den untersuchten Gruppen mit dem Mann-Whitney-Test verglichen. Nach logarithmischer Transfromierung der RQ-Werte wurde eine Pearson Korrelation durchgeführt. Von 12 Tumorproben wurde der epigenetische Status der Subtelomere für das Chromosom 2p durch Bisulfit-Sequenzierung bestimmt.

Die Expression aller TERRA Moleküle war um das 14 und 31-fache bei GBM niedriger verglichen mit jeweils AA-und DA ($p < 0,05$). Zelllinien der Astrozytome zeigen TERRA Expression vergleichbar wie bei GBM, im Gegensatz dazu zeigt die ALT-Zelllinie ähnliche Expression wie bei DA. Chromosom 2p-und 18p-TERRA Expression ändert sich nicht signifikant mit dem Tumorgrad. Die durchschnittliche Telomerlänge war bei niedriggradigen Tumoren DA (22 ± 15 kbp) und AA (17 ± 5 kbp) deutlich erhöht, verglichen mit GBM (9 ± 4 kbp). Die gesamte TERRA Expression korreliert mit der Telomerlänge (Pearson-Koeffizient $P = 0,38$, $p = 0,01$). Tumor Proben zeigen Unterschiede der Durchschnittswerte der CpG-Methylierung im 2pTERRA-Promotor von 89% und 83% bei jeweils niedrigen und hohen 2p-TERRA Expressionswerten. Erste Analysen zeigen einen signifikanten Zusammenhang zwischen dem Überleben der Patienten und der gefundenen TERRA Expression. Niedrige Expression von TERRA korreliert mit einer schlechten Prognose.

Die relative Expression von TERRA korrelliert in astrozytären Tumoren signifikant mit der Telomerelänge, mit dem Tumorgrad und dem Methylierungsstatus des Promotors. Die relative TERRA Expression könnte ein vielversprechender Kandidat als diagnostischer und prognostischer Marker bei astrozytären Tumoren sein.

1 Abstract

Telomeres are localized at chromosomal ends to protect and stabilize the whole genome from deterioration. A telomere consists of TTAGGG repeat DNA sequences. It has been shown recently, that telomeres are transcribed into telomeric repeat-containing RNA (TERRA) involved in telomere maintenance mechanisms (TMM) and chromatin organization. TERRA transcription originates in the subtelomere and depends on the epigenetic state of the telomere. Astrocytomas utilize two TMM based on telomerase activity (TA) and on alternative lengthening of telomeres (ALT). ALT results telomeres longer than TA mechanism. Correlation between TMM and patient survival was demonstrated for high grade astrocytomas, especially glioblastoma multiforme (GBM). This finding and dismal prognosis of patients suffering from GBM emphasizes the need for detailed characterization of telomere length and TERRA in these tumors.

A panel of tumor tissue samples (ts) were analyzed which originated from 46 patients with diagnoses astrocytomas WHO grade II to IV: 12 diffuse astrocytomas (DA) grade II, 6 anaplastic astrocytomas (AA) grade III and 28 GBM grade IV. Further, human tumor cell lines were analyzed, 12 originated from astrocytomas and one ALT cell line from an osteosarcoma. RNA and DNA were isolated. Relative quantities (RQ) of transcript expression levels for overall and chromosome-specific TERRA were measured by reverse transcription following real-time PCR and compared to beta actin and 36B4 levels. Telomere length was determined by TRF Southern blotting and real-time PCR. RQs were compared between groups by Mann-Whitney test and log-transformed for Pearson correlation calculation. Epigenetic state of subtelomeric region for chromosome 2p was determined by bisulphite allelic sequencing of 12 ts.

Overall TERRA expression was decreased 14 and 31-fold in GBM compared to AA and DA ($p<0.05$), respectively. Astrocytoma cell lines contained TERRA levels found as in GBM, the ALT cell line as in DA. Chromosome 2p- and 18p-TERRA expression was not significantly altered by grade. Mean telomere length was significantly increased in low grade tumors DA (22±15 kbp) and AA (17±5 kbp) as compared to GBM (9±4 kbp). Overall TERRA expression correlates with telomere length (Pearson's coefficient P=0.38; p=0.01). Tumor samples with low and high 2p-TERRA expression levels demonstrate different average CpG methylation at 2p-TERRA promoter of 89% and 83%, respectively. Primary analysis of expression and survival data indicates a significant connection of overall survival and expression of TERRA. Low TERRA levels correlate with a poor prognosis.

Resulting data demonstrate that TERRA levels are in astrocytomas significantly related to telomere length, tumor grade and to promoter methylation status. Expression levels of TERRA can be considered promising candidates as diagnostic and potentially prognostic markers in astrocytomas.

2 Introductions

2.1 Glioblastoma multiforme

2.1.1 Gliomas

Gliomas are a group of central nervous system (CNS) neoplasms with distinct histologic characteristics, comprising almost 60% of total human CNS malignancies. The common forms of gliomas in humans are astrocytomas, and the most aggressive form of astrocytomas is glioblastom multiforme (GBM) with histological signs of neoangiogenesis (Figure 2.1). GBMs are the most common form of malignant brain tumours in adults and it is also the most deadly one. The median life expectancy is about twelve month and only less than 5% of patients are still alive five years after diagnosis. The biology of gliomas is unique. Systematic metastasis occurs, but is very rare in this type of tumours. The majority of glioblastomas recur within two centimetres of their original tumour margin. They are very aggressive and frequently have been referred to as “whole-brain” or “whole-CNS” disease and a total resection almost always failed.[3]

Malignancy grade is a prognostic factor for gliomas, although biological behaviour of gliomas of same grade is not always predictable. The period of survival and the choice of treatment depend on the malignancy grade. [2]

Astrocytomas are found in young adulthood, but have a peak incidence in the third and fourth decade of life [4].

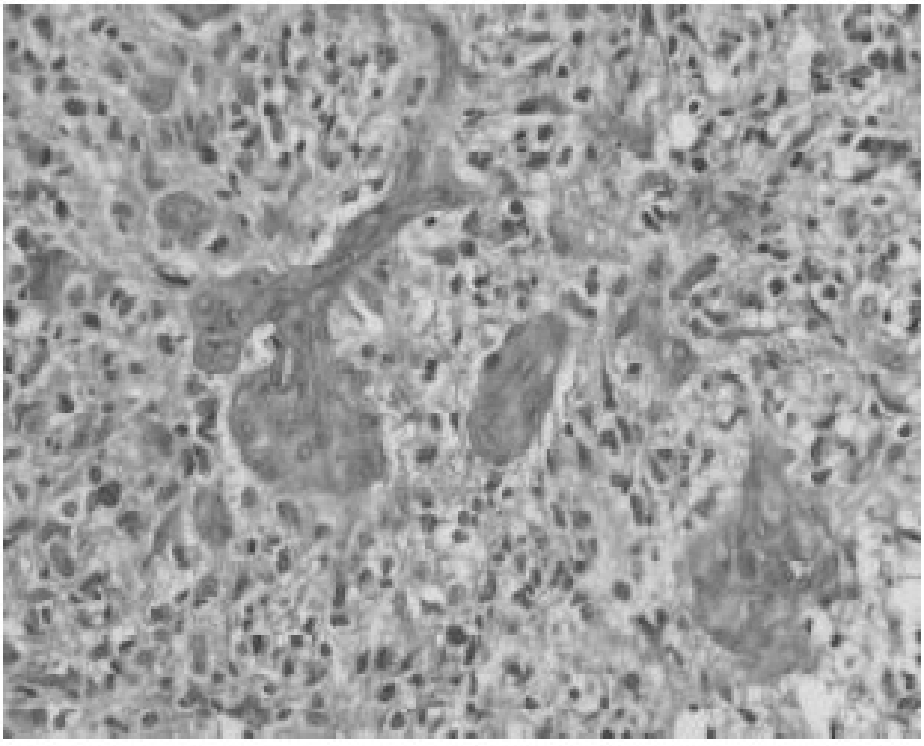


Figure 2.1 **Vascular proliferation in glioblastoma multiforme [2]**

Gliomas are classified into

- astrocytomas,
- oligodendrogliomas,
- oligostrocytomas and
- ependyomas (see table 2.1)

based on the presumed cell of origin. They are further classified according to their degree of malignancy. High grade gliomas can manifest de novo (primary) or develop from a previous low grade tumour (secondary) (Fig. 2.2). About two thirds of the low grade gliomas progress to high grade tumours [2]. Seventy percent of grade II gliomas transform into grade III and IV tumours within 5 – 10 years. [5]

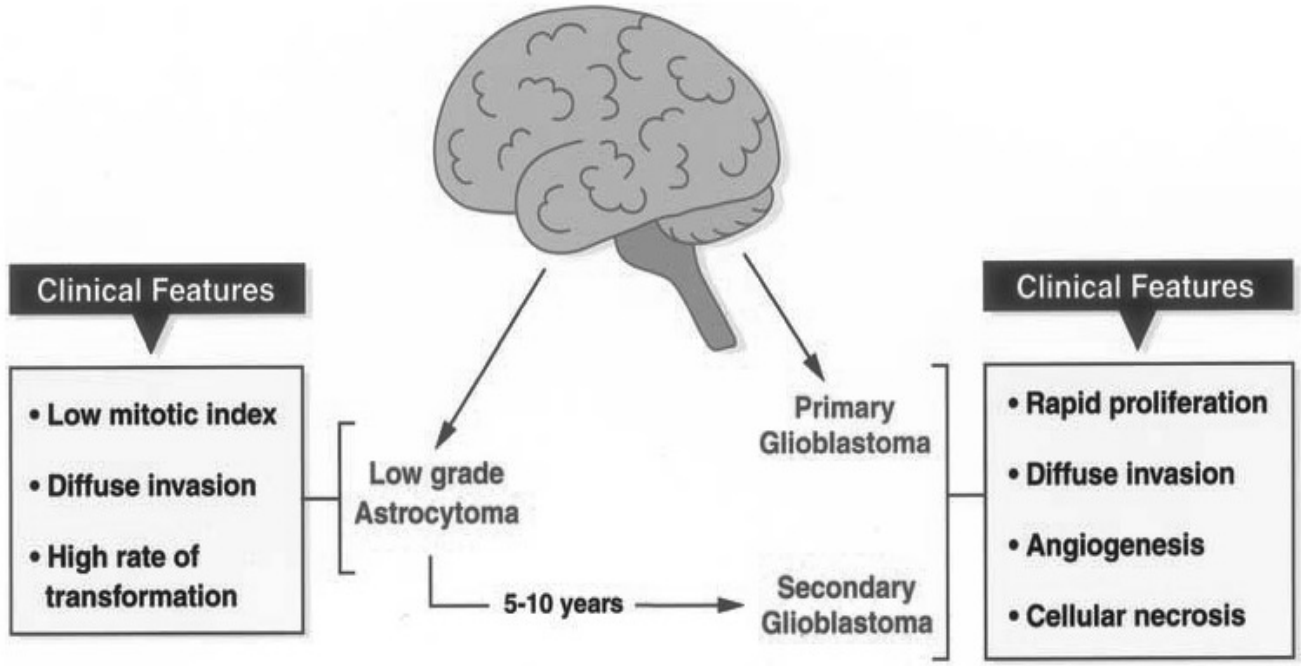


Figure 2.2 **Two pathways to GBM** GBM can develop over 5 to 10 years from low-grade astrocytomas (secondary GBM), or it can be the initial pathology at diagnosis (primary GBM). [5]

2.1.2 WHO grading system

There are different grading systems applied for astrocytomas. One widely used system is the World health organisation (WHO) system. The main histological signs of malignancy in this system are nuclear atipia, mitotic activity, vascular proliferation and necrosis (see table 2.2). Presence of mitotic activity stands for poor prognosis of these tumours. Accurate pathological grading is essential because it defines treatment and prognosis. The histological features of the tumour and patients age are major prognostic factors and have more influence than any specific therapy on the outcome.

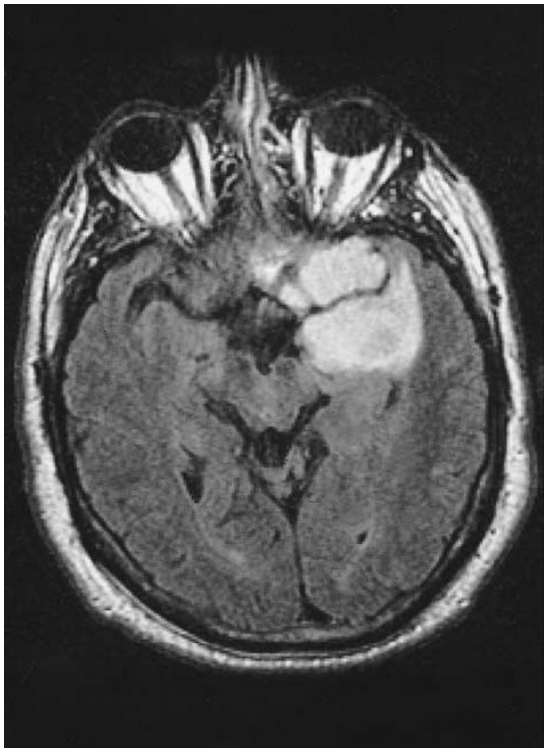


Figure 2.3 **Magnet resonance image (MRI) of low grade astrocytoma**

WHO Classification of Glial Tumours (2000)	Prevalence among primary CNS tumors (%)	Mean age of occurence (years)	Mean survival (years)
1. Astrocytic tumours			
1.1. Diffusely infiltrating astrocytomas	>60		
1.1.1. Diffuse astrocytoma:		34	3.7–8
– fibrillary			
– protoplasmic			
– gemistocytic			
1.1.2. Anaplastic astrocytoma		41	2–5
1.1.3. Glioblastoma:	40.6	53–54	<1
– giant cell			
– gliosarcoma			
1.2. Pilocytic astrocytoma	1.5		
1.3. Pleomorphic xanthoastrocytoma			
1.4. Desmoplastic cerebral astrocytoma of infancy			
1.5. Subependymal giant cell astrocytoma			
2. Oligodendroglial tumors	2–8	42.8	
2.1. Oligodendroglioma		36–42.6	3.5–9.8
2.2. Anaplastic oligodendroglioma		45–48.7	3.9–5.3
3. Mixed gliomas			
3.1. Oligoastrocytoma		36.5	3.0–7.1
3.2. Anaplastic oligoastrocytoma		38–45	1.1–4.3
4. Ependymal tumors	1.7–3.0		
4.1. Ependimoma:			
– cellular			
– papillary			
– clear cell			
– tanycytic			
4.2. Anaplastic ependymoma			
4.3. Mixopapillary ependymoma			
4.4. Subependymoma			
5. Glial tumors of uncertain origin			
5.1. Astroblastoma			
5.2. Gliomatosis cerebri		40–50	
5.3. Chordoid glioma of the 3rd ventricle			

Table 2.1 **Glial tumour classification, incidence, patient age and survival [2]**

The distinction between an anaplastic astrocytoma (grade III) and glioblastoma multiforme (grade IV) depends on the

- microvascualar proliferation or
- tumour necrosis

In addition, many cases of GBM arise by a different genetic pathway than anaplastic astrocytomas. GBMs are largely a disease of adults, although cases in children are also known. The incidence of GBM increases with age and is highest at age of 65 to 75 years, and than it declines. Men are affected more commonly than women with a ration of 1.6:1

WHO 2000		St. Anne-Mayo 1988		Ringertz	Kernohan, 1949
name	grade	description	grade		
Pilocytic astrocytoma, subependymal giant cell astrocytoma	I	Absence of four major criteria: a) nuclear atypia b) mitoses c) endothelial proliferation d) necrosis	1		
Diffuse astrocytoma	II	1 feature (frequently a)	2	Astrocytoma	1
Anaplastic astrocytoma	III	2 features (a+b)	3	Intermediate astrocytoma (with focal anaplasia)	2
Glioblastoma	IV	3 features (a+b+c+/–d)	4	Glioblastoma	3 ir 4

Table 2.2 **Grading systems for astrocytic gliomas [2]**

- Grade I tumours are biologically benign and can be surgically cured-
- Grade II tumours are low-grade malignancies that may follow long clinical courses but are not curable by surgery.
- Grade III tumours are malignant and lead to death within a few years.
- Grade IV tumours are highly malignant, usually recalcitrant to chemotherapy and lethal within 9-12 months. Standard therapy at the AKH Vienna is surgery with concomitant chemo- and radiotherapy with akylating agents (1995-2000 CCNU or fotemustine/dacarbacine) and since 2000 Temozolamide (personal communication C. Marosi)

The central nervous system (CNS) consists of two major cell types:

- neurons and
- glia

The latter are subdivided into three subpopulations:

- astrocytes
- oligodendrocytes
- microglia

Astrocytes are the most abundant type of glia cells in the CNS and provide growth factors for neurons. They have some very important functions, for example supporting and protecting neurons, inducing neurogenesis, regulating synapse formation and they initiate immune responses.

Oligodendrocytes synthesize myelin in the CNS and also provide growth factors for neurons. A subgroup of gliomas is characterized as astrocytomas because the neoplastic cells show morphological characteristics of astrocytes. These tumours express differentiation markers of astrocytes, such as intermediate-filament glial fibrillary acid protein (GFAP) and calcium binding protein S100ß. This makes them different from the other main subgroup of gliomas, the oligodendrogliomas and makes a connection between normal glial differentiation and glial malignancy.

Progenitor cells and malignant cells share some common features: simplicity in morphology, extended proliferation capacity, potential to differentiate and ability to migrate over longer distances. It is a widely accepted idea, that malignancy represents an undifferentiated state of their cellular origins. Progenitor and malignant cells share some characteristics. Both show a simplicity in their morphology, extended proliferation capacity, the ability to migrate over longer distances and the potential to differentiate [3].

2.1.3 Molecular genetic changes

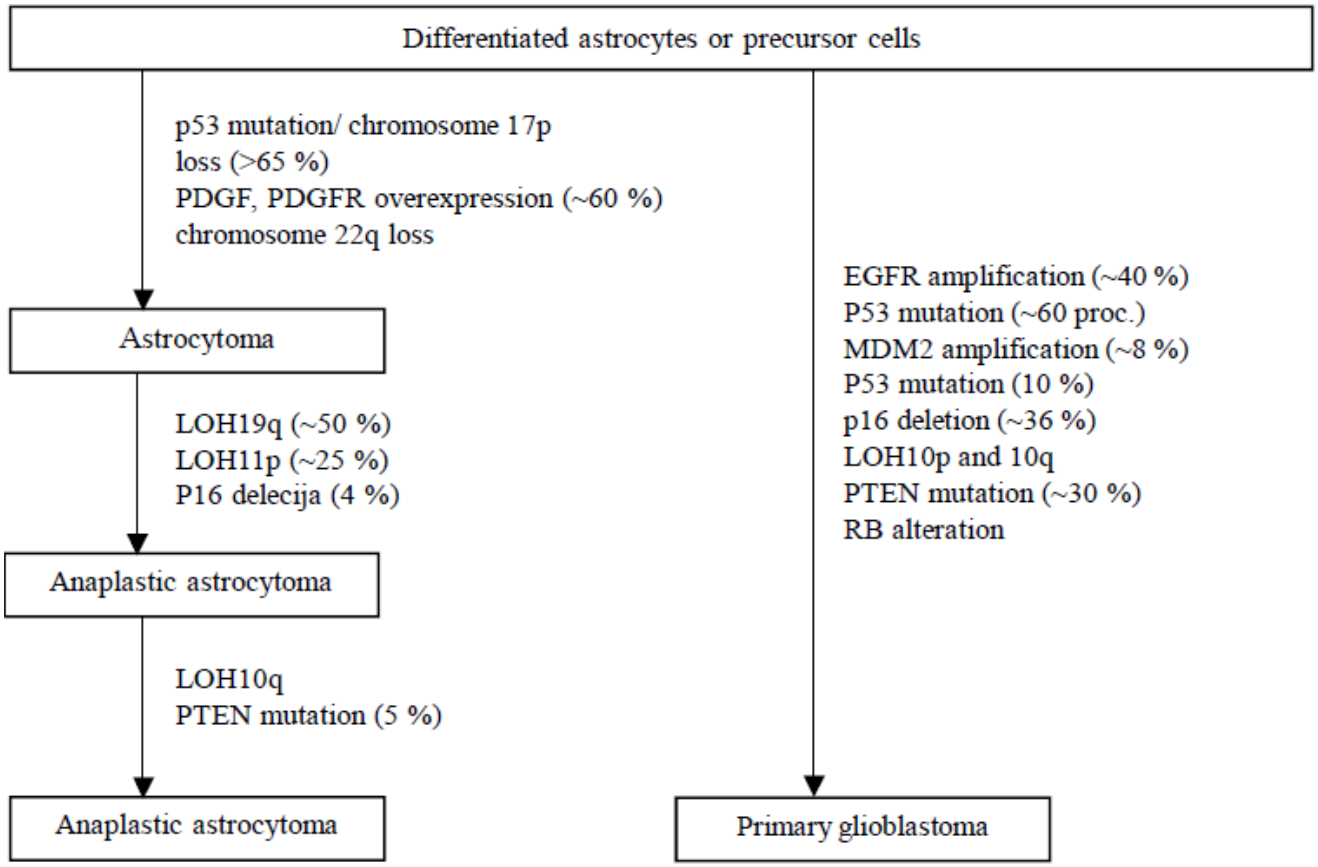


Figure 2.4 **Molecular genetic changes in astrocytic brain tumours [2]**

p53, p16, PTEN ….tumour suppressors

EGFR … epidermal growth factor receptor

RB … retinoblastoma protein

PDGF … platelet derived growth factor

PDGFR … platelet derived growth factor receptor

MDM2 … negative regulator of p53 tumour suppressor

LOH … loss of heterozygosity

Diffuse astrocytoma WHO grade II progress to anaplastic astrocytoma and glioblastoma, genetic changes occur which are often different from the changes found in a primary (de novo) glioblastoma. The genetic changes are shown in Figure 2.4

It could be shown, that younger age is significantly correlated with a better outcome. Among molecular parameters, TP53 mutations predict a favourable clinical course independent of patient age and primary or secondary GBM status. LOH10q in GBM is associated with poor survival. Loss of either LOH1p or LOH19q has no prognostic significance in GBMs, but the combined loss of both of the two chromosomal arms may identify a patient group with better prognosis and potentially sensitive to chemotherapy. Loss of LOH19 is found in both, primary and secondary GBMs, but shows a tendency to be more common in primary GBMs [6].

2.2 Telomeres

2.2.1 Structure and function of telomeres

Telomeres protect the chromosomal ends from being recognized by DNA repair mechanism as double strand breaks, they prevent them from chromosomal end fusion and degradation. In this way they maintain the stability of the linear chromosomes.
They were first described by Hermann Muller in the fruit fly *Drosophila melanogaster*. He was the first to coin the term "telomere" in 1938. He predicted that telomeres must have a special function in sealing the ends of chromosomes. A chromosome could not persist indefinitely without telomeres. [7]

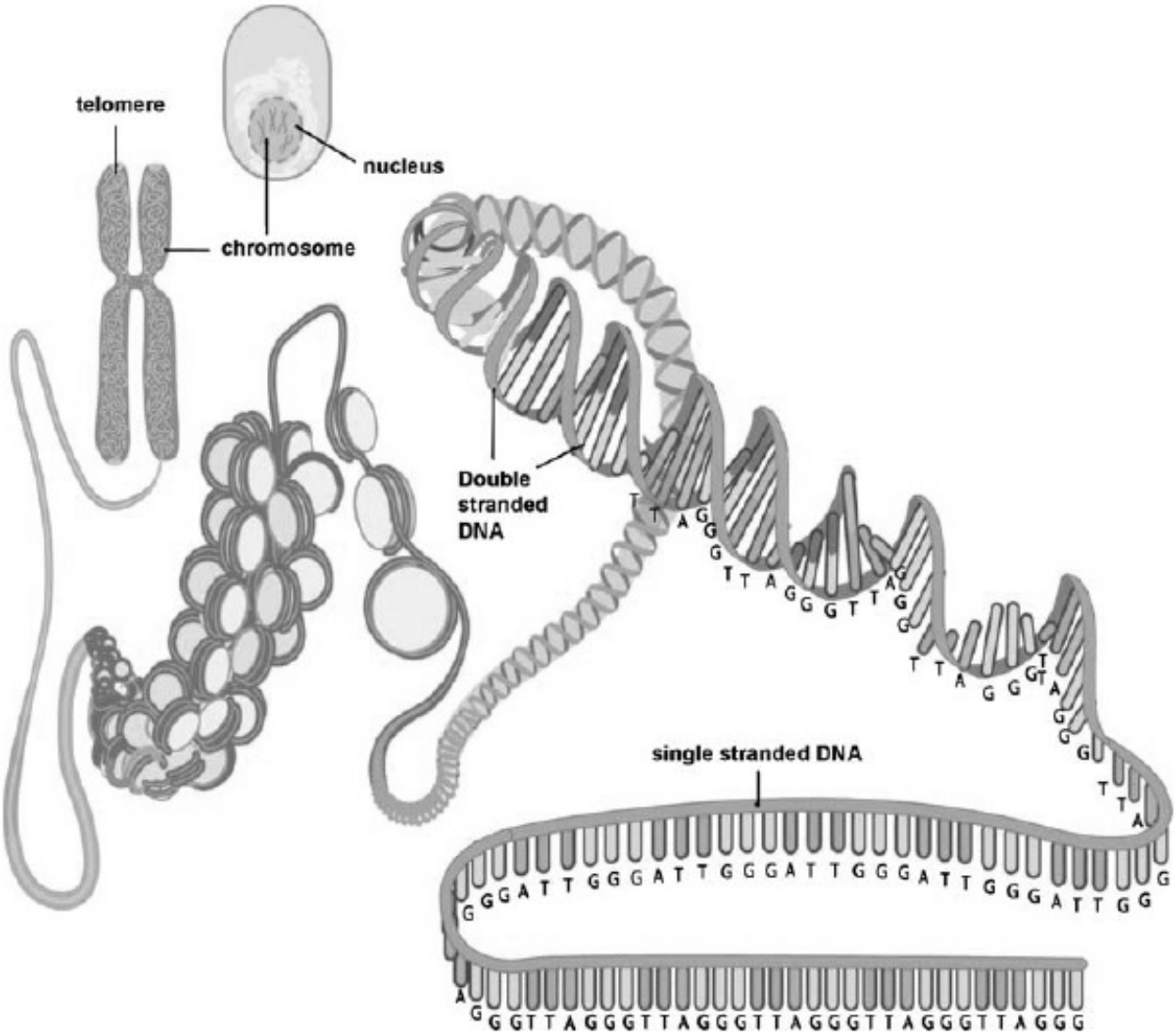


Figure 2.5 **Telomeres are located at the ends of the linear chromosomes and consists of TTAGGG repeats in human [8]**

Telomeric DNA sequences are very similar among otherwise widely divergent eukaryotes. The essential telomeric DNA consists of a stretch of very simple tandem repeated sequences. Chromosomal ends, the telomeres, are composed of thousands of repeats of a short 6 base pair (bp) sequence element. For vertebrates, for example, telomeric sequences consists of TTAGGG repeats (Figure 2.5 and 2.6) [9].

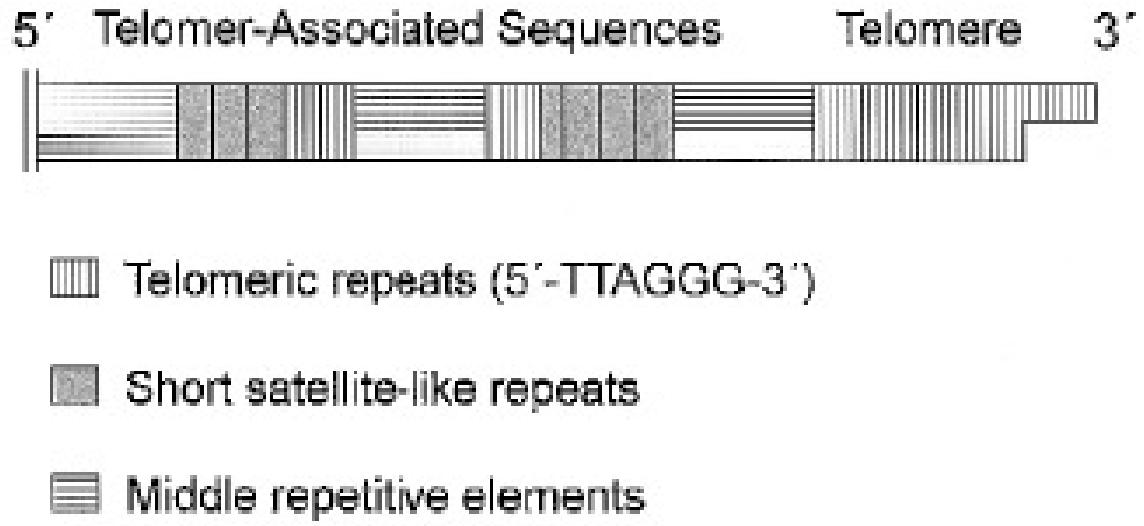


Figure 2.6 **Organization of eukaryotic telomeres.** Telomeres consist of repetitive telomeric sequences with single-stranded 3´ overhangs. Subtelomeric regions are composed of various short satellite-like repeats and middle-repetitive elements, interspersed with telomeric repeats. [10]

The G-tail is protected by the so-called shelterin complex, consisting of 6 proteins. The telomeric repeat binding factor 1 (TRF1) and 2 (TRF2), which bind double stranded parts of the telomeric DNA. The telomere – shelterin complex forms two internal loops, the D-loop and the T-loop (Figure 2.7). POT1, another protein of the shelterin complex is binding the single stranded part of the telomeres and is suggested to be important for forming the D-loop. This complex protects the telomere from erosion and end-to-end fusion. Beside this 3 DNA-binding proteins the telomere shelterin complex contains other proteins, like repressor activator protein 1 (Rap1), TPP1 and TRF1-interacting nuclear factor 2 (TIN2). Telomere shortening results in chromosome destabilization and inability to recruit proteins of the shelterin complex. The T-loop can not be formed and chromosome ends get uncapped. This leads to double strand breaks and may lead to inactivation of the p53 and p16/Ink4a pathway and may result in senescence or apoptosis[8].

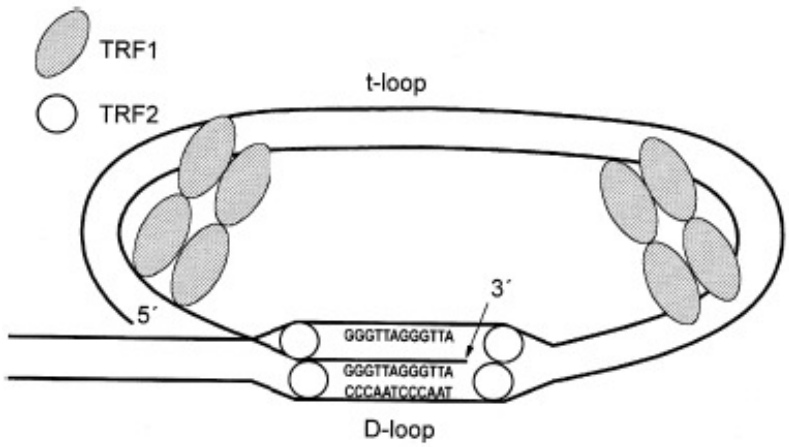


Figure 2.7 **Formation of telomere loops.** The single-stranded 3´-overhang of the very ends of telomeres invades upstream double-stranded telomeric sequences and hybridizes to complementary sequences, thereby forming a **displacement loop (D-loop)**. TRF2 is supposed to stabilize this structure. Telomeric DNA loops out and might be stabilized by TRF1 proteins. [10]

50 – 100 bp of telomere sequences are lost with every cell cycle form every chromosomal end, due to the end-replication problem (Figure 2.8). The inability of DNA polymerase to replicate the 3´-ends of chromosomal DNA leads to this gradually shortening and causes the loss of ability of telomeres, to protect the chromosomal ends. These unprotected ends take part in end-to-end chromosomal fusion, leading to crisis and death of the cells.

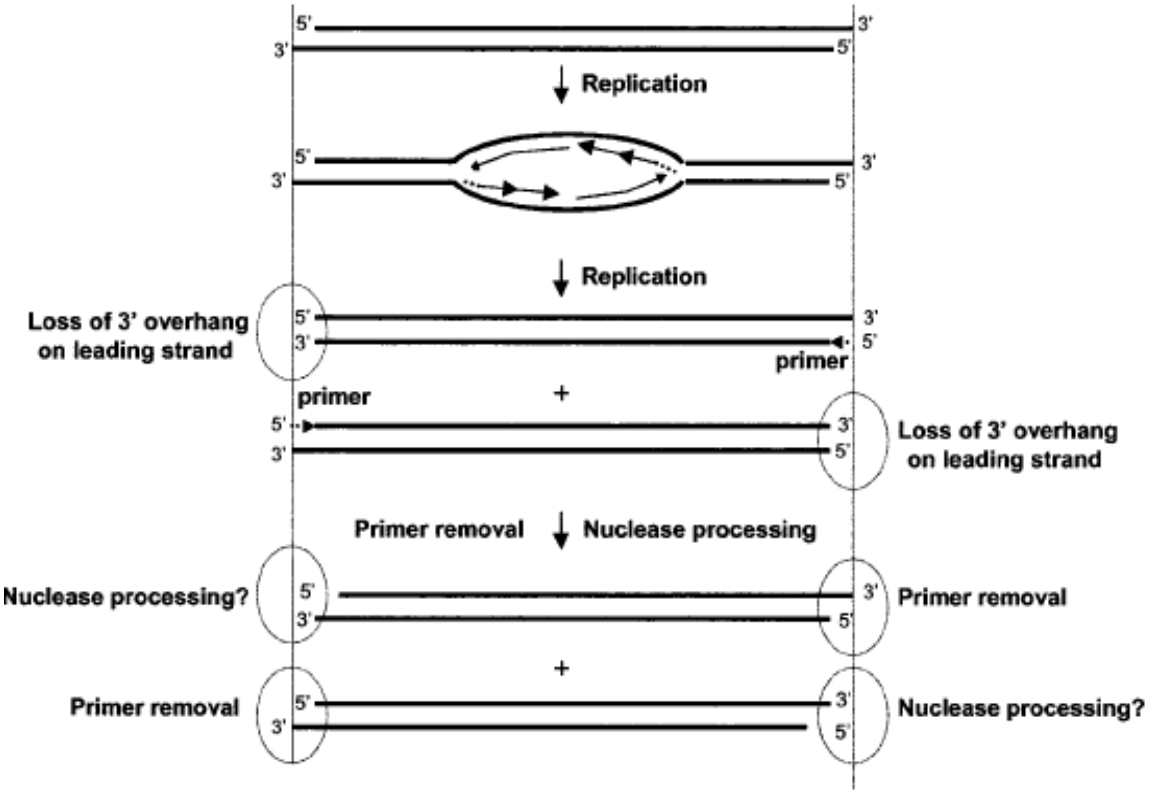


Figure 2.6 **End replication problem.** DNA replication by the conventional polymerase proceeds in the 5´-to-3´ direction. The newly synthesized leading strands would not generate overhangs, but the newly synthesized lagging strands would lose their extreme 3´ end after RNA primers are removed. In addition, both parental strands might also be subject to nuclease processing. [11]

2.2.2 Maintaining of telomeres

Almost all types of malignant cells maintain their telomeres by telomere-maintaining mechanisms. 85 – 90 % of them maintain their telomeres by the enzyme telomerase, a ribonuclear protein complex consisting of an RNA moiety (hTR or alternatively termed hTERC) which serves as template for telomeric repeats, and the catalytic subunit (hTERT) which has a reverse transcriptase function. The telomerase-negative remaining malignant cells have invented mechanisms, termed alternative lengthening of telomeres (ALT), which is based on recombination events leading to interchromosomal exchanges of sequence information. Both mechanisms are strongly suppressed in most of normal somatic human cells, to prevent a unlimited replicative potential and immortality [12].

2.2.3 Dysfunction of telomeres

Telomeres can become dysfunctional through a variety of mechanisms. The most common is shortening due to the end-replication problem. The 3´- end of linear chromosomes cannot be completely replicated (Figure 2.8). So, telomeres get shorter with every replication cycle. When they become critically short, they fail to cap and protect the chromosome ends, leading to replicative senescence or apoptosis [13]. Telomeres can also loose function as a result of direct damage mainly by cellular stress, e.g. they are susceptible to oxidative DNA damage, which is a major source of DNA damage in mammalian cells [14].
Mutations that alter expression or function of telomere-associated proteins can also cause the dysfunction of telomeres.

2.2.4 DNA damage response

Dysfunctional telomeres are sensed as DNA contains a double strand break (DSB). So, they induce a classic DNA DSB response. These telomeres are marked by gamma-H2AX and a variety of other DNA damage response proteins, and are so similar or identical to unrepaired DNA breaks[15]. Dysfunctional telomeres, similar to irreparable DNA damage, are for a organism a great risk for developing cancer. They elicit cellular tumour suppressor mechanisms like apoptosis and senescence. Whether cells undergo apoptosis or senescence depends on cell type and genetic background, for example the p53 status of cells. Apoptosis eliminates cells, and senescence arrests their growth permanently [16].

Both are controlled by p53 tumour suppressor protein. p53 is a transcriptional regulator that activates or represses genes in response to stress [17]. These p53 target genes stimulate DNA repair, transient or permanent cell cycle arrest (senescence) or cell death (apoptosis), depending on cell type, degree and type of damage. Cells that lack normal p53 regulation or function show a tendency to die in response to telomere dysfunction. Some normal human cells, on the other hand, undergo a senescence growth arrest. In either case, if present, p53 is essential for mediating the cellular response to telomere dysfunction [18].

2.2.5 Impact of telomeres on cancer

Cellular tumour suppressor mechanisms ensure that cells with telomere dysfunction can not divide. Cells that have lost these mechanisms of apoptosis and senescence, for example owing to mutations in p53 pathway and develop dysfunctional telomeres are at a great risk for neoplastic transformations. Even cells that have lost p53 must acquire a way to stabilize telomeres and so prevent loss of genetic information. Most frequently is this achieved by expression of telomerase, but there exist alternative pathways like ALT [19]. Telomeres can so both suppress and facilitate cancer.
Summing up, functional telomeres are essential for prevention of genomic instability (Fig.2.9). By loosing telomeres, the cellular tumour suppressor mechanisms apoptosis and senescence are engaged. These responses prevent cells with dysfunctional telomeres from cell division to reduce the risk of cancer.

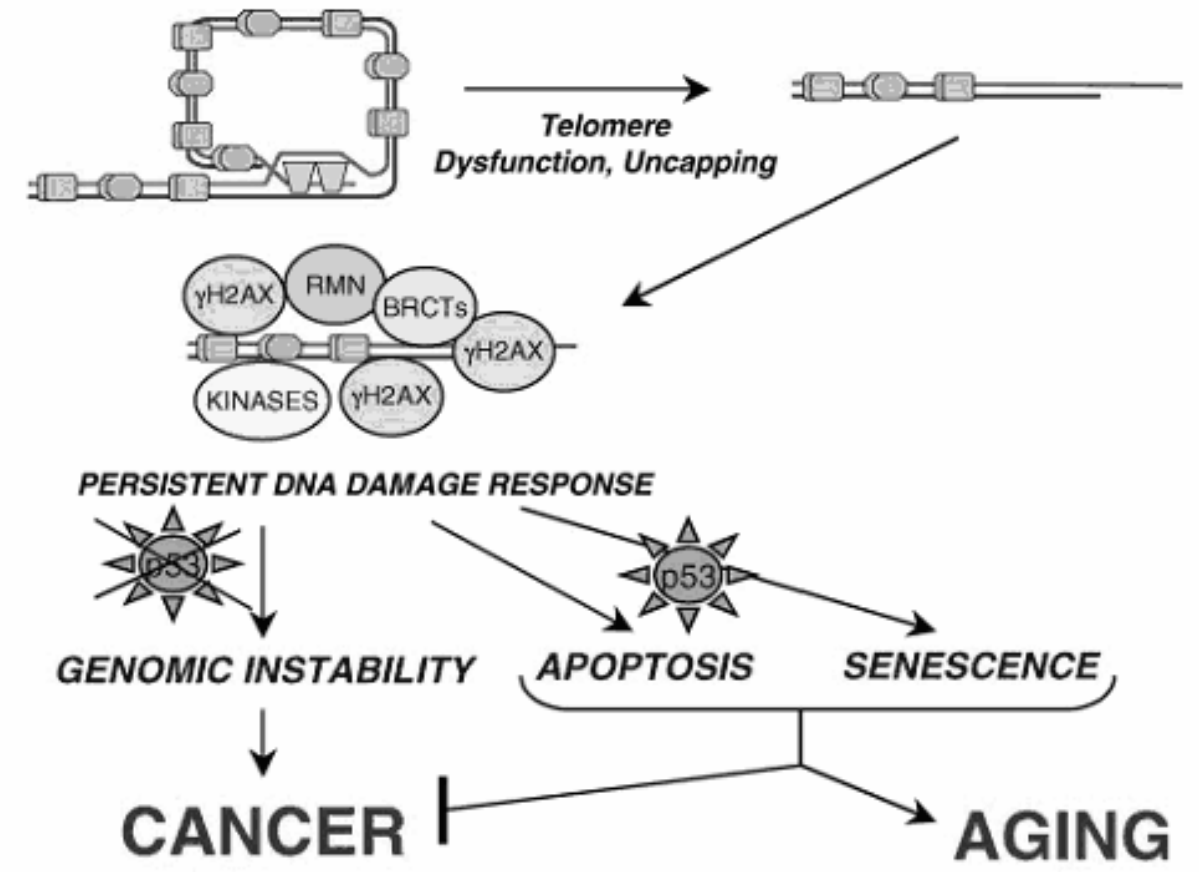


Figure 2.9 **The outcome of telomere dysfunction includes the generation of a DNA damage signal**. The fate of cells showing this signal, senescence or apoptosis, depends on p53. This cell fate influences the organism phenotypes of cancer and aging [13].

2.3 Expression of telomeres

2.3.1 Telomeric repeat containing RNA (TERRA)

Historically based on chromosome staining methods, telomeres could be classified as non-transcribed heterochromatin. They are enriched in heterochromatin marks like:

- trimethylation at lysine 9 on histone H3
- trimethylation at lysine 20 on histone H4 and
- HP1 (Heterochromatinprotein 1)

The heterochromatic state, the geneless nature of telomeres, and the fact, that experimentally inserted reporter genes to the subtelomeric region led to the idea of transcriptionally silent telomeres.

Contrary to the idea, of telomeres being silent genomic regions, recently it has been discovered that mammalian telomeres are transcribed by RNA polymerase II into TElomeric Repeat containing RNA (TERRA) molecules, which represent a new class of mammalian

non-coding RNAs [20, 21]. They range in size from 100 nt to 9000 nt and they are only present in the nuclear fractions. TERRA is evolutionary conserved, because it is found in different human cell lines as well as mouse, hamster and zebrafish. Not only the telomeres, but also the subtelomeric region is transcribed and TERRA molecules consist of subtelomeric derived RNA and telomeric UUAGGG repeats (Figure 2.9). *In silico* analyses and experimental data validated that TERRA transcription starts form different subtelomeres towards the chromosome ends.

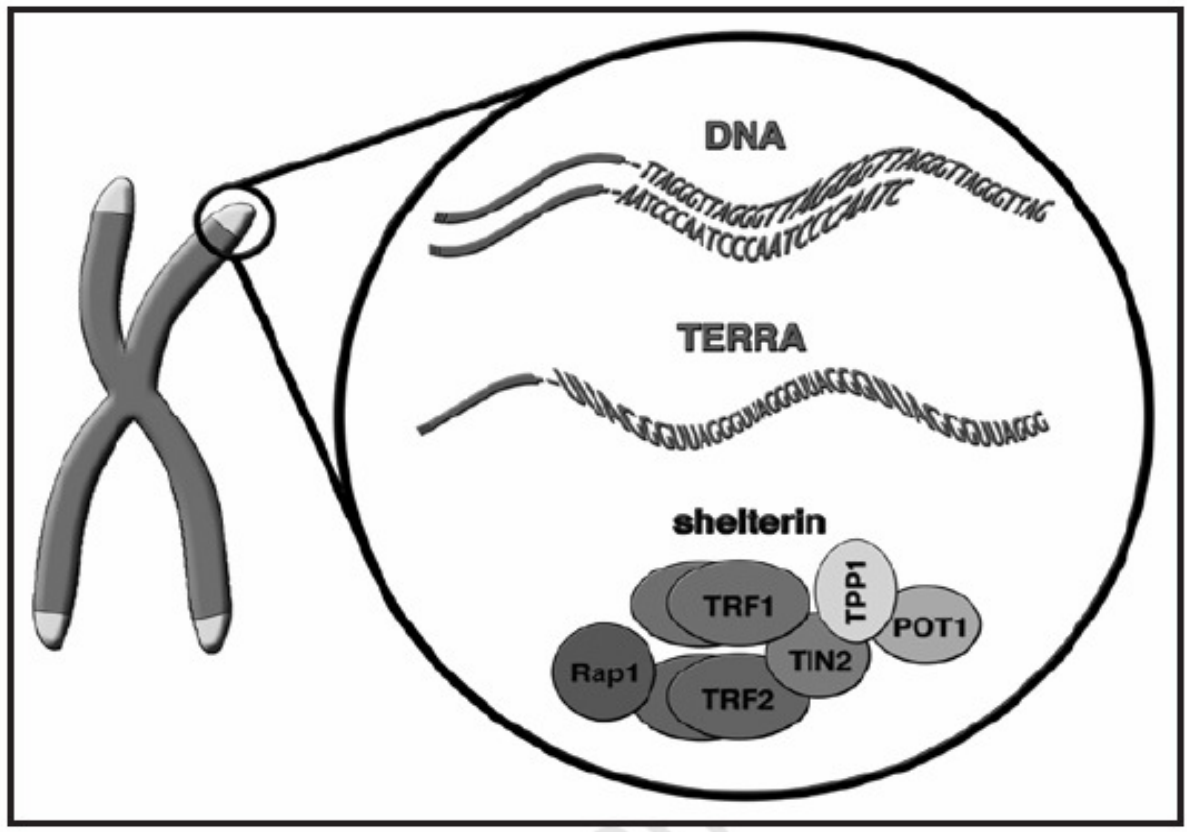


Figure 2.9 Mammalian telomeres comprise $(TTAGGG)_n$ DNA sequences, $(UUAGGG)_n$ –containing RNA molecules (TERRA) and the shelterin protein complex (TRF1, TRF2, POT1, TIN2, TPP1 und Rap1). Many additional telomere interacting protein are not shown in this figure. [7]

While the length distribution of TERRA molecules does not vary substantially among different mammalian cell lines, the amount of TERRA is variable. There exist many evidences that the amount of TERRA correlates with telomere length. This suggests a cell- or tissue-specific regulation of TERRA expression.
Tumour tissue shows often a low level of TERRA molecules compared to healthy tissue, leading to the idea TERRA could be down regulated during cancer development in vivo [20] . However, up to now, very limiting information about TERRA expression in clinical samples is available.

TERRA molecules can be recovered exclusively in nuclear fractions, but only 7% of TERRA molecules were found in polyA+ nuclear RNA fractions, while 62-64% of transcripts for coding genes like c-myc and actin were detected. Most of the TERRA molecules are not poly adenylated. Interestingly, almost all polyA+ TERRA molecules are larger than 2kb, indicating that short TERRA molecules are abundant and not polyadenylated [21] .

2.3.2 TERRA at telomeres

TERRA molecules form discrete nuclear foci that overlap with telomeric heterochromatin in interphase cells as well as inactive metaphase cells. The ability of TERRA molecules to interact with telomeres independently of their transcription indicates that TERRA is an integral component of telomeric heterochromatin and suggests that TERRA might participate in maintaining telomeric architecture. TERRA might be involved in maintaining the heterochromatic state of telomeres [7].
Because TERRA is detected in only a subset of telomeres, it could be not an essential and permanent part of telomeric chromatin, but rather has a transient structural function during assembly of telomeres. Alternatively TERRA could have telomeric-state-dependent functions, for example, in the establishment of telomeric heterochromatin or in the regulation of telomerase.
TERRA remains associated with telomeres, but it has not been shown whether TERRA molecules can move between telomeres or if it remains associated to the telomere, it is transcribed from.
It exist two possible means, by which TERRA could be tethered to telomeres. One is through interaction of a telomeric protein with TERRA or, second, by direct interaction of the RNA with telomeric DNA. Both options are possible. Up to now, there is no evidence, that telomeric proteins are involved in binding of TERRA to telomeres.

2.3.3 Regulation of TERRA

2.3.3.1 Non-sense-mediated decay machinery

Regulatory pathways exist for both: the abundance and the cellular localization of TERRA. For example non-sense-mediated decay (NMD) machinery, which is degrading mRNAs containing pre-mature termination codons, has an essential function in displacing TERRA from telomeres. Telomere sequences contain termination-codons within the repeat sequence. When proteins of NMD machinery were depleted by RNA interference (RNAi), TERRA foci on telomeres were strongly increased. This shows a regulating effect of these proteins of

TERRA on telomeres. But total levels of TERRA and its half-life were not affected by depleting NMD proteins. It is possible, that NMD proteins interact directly with TERRA on telomeres.

There exist two possibilities, by which TERRA abundance could be diminished at telomeres. First, NMD proteins could be required for the displacement of TERRA from telomeres, without influencing the degradation. Second, they are involved in local TERRA degradation at the telomere. [22]

2.3.4.2 RNA interference

RNA interference (RNAi) is a highly conserved mechanism for gene silencing triggered by small interfering RNA molecules (siRNA). These molecules are 22 nucleotides in length and double stranded (ds) generated by degradation of long ds RNA molecules by the ribonuclease III enzyme Dicer (Fig.2.10).

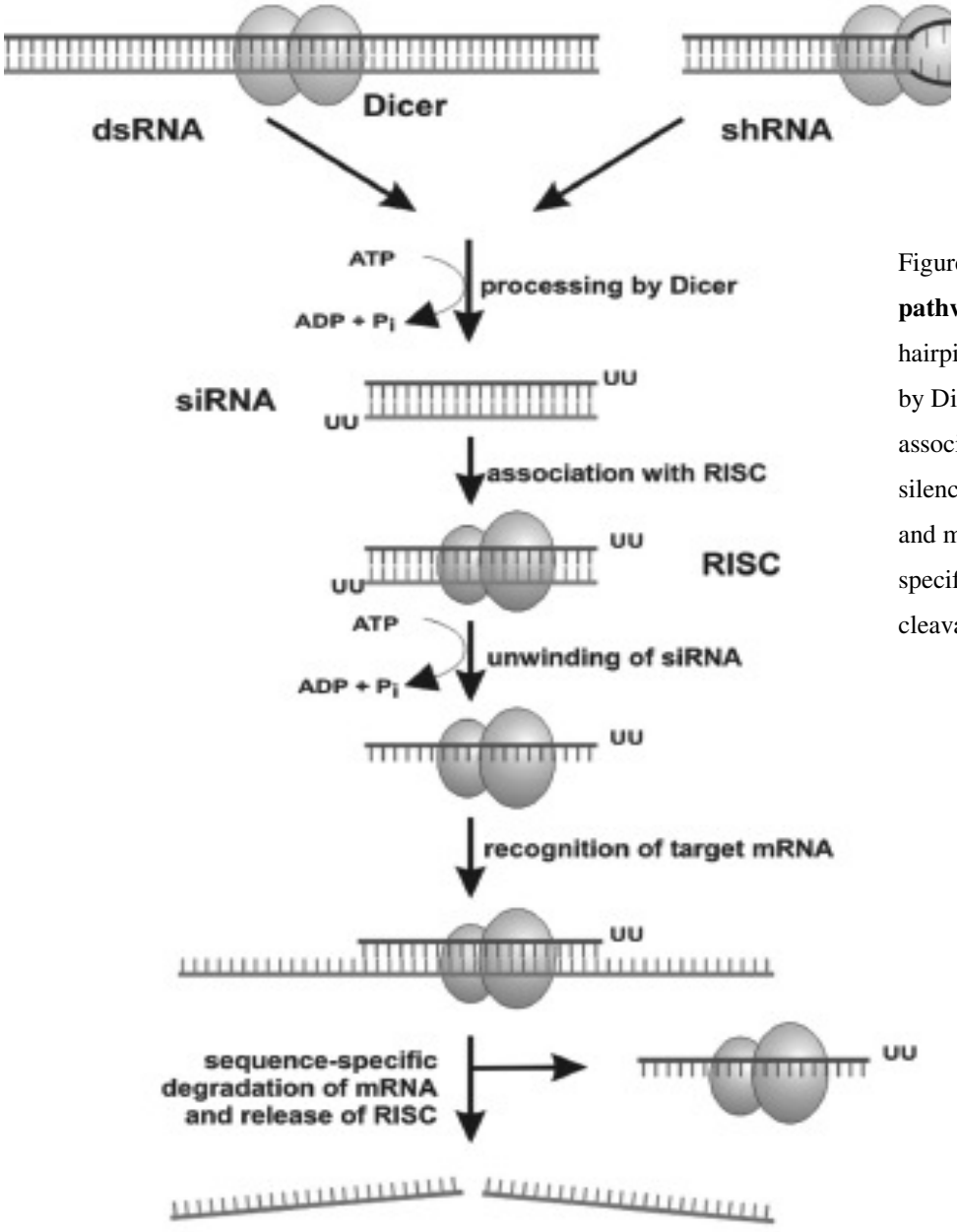


Figure 2.9 **RNA interference pathway.** Long ds RNA or small hairpin RNA (shRNA) is processed by Dicer to form a siRNA, which associates with RNA-induced silencing protein complex (RISC) and mediates target sequence specifity for subsequent mRNA cleavage. [1]

It has been shown, that RNAi machinery could be directly involved in heterochromatinization of telomeres in yeast. It is possible, that telomere transcription triggers telomere heterochromatinization by RNAi, and TERRA serves as precursor RNA to generate telomeric siRNA.

Two lines of evidence exist for RNAi function of TERRA molecules:

- When mouse cells are transfected with synthetic RNAi, Argonaut1, one of the proteins of the RNAi machinery, is increased associated with telomeres and TERRA are overproduced [23].
- Mouse embryonic stem (ES) cells deficient for Dicer show a low TERRA level when compared with wildtype (wt) counterparts [20].

2.3.4.3 Chromatin state

As mentioned before, telomeres show some typical features of heteochromatin. Mouse cells deficient for histone methyl transferases SUV39H1/SUV39H2 and SUV4-20H1/ SUV4-20H2 have a decreased level of histone methylation at telomeres and aberrant telomere length. Additionally, progressive shortening of telomeres induces a decrease in density of trimethylation of histone H3 at lysine 9 and on histone H4 at lysine 20 at telomeres and methylation of the subtelomeric region is also decreased. This indicates that the structure of mammalian telomeres is regulated epigenetically and this could also be involved in TERRA regulation. [7]

2.3.4 Functions of TERRA

2.3.4.1 Telomerase

As mentioned in 2.3.3.1, TERRA is displaced in human cells at telomeres by NMD factors. NMD factors interact directly with telomeric chromatin [21]. One of these factors, EST1A/SMG6 was identified due to its sequence-similarity with Est1, a telomerase-associated protein in S.cerevisiae. Est1 directly binds RNA moiety of telomerase and to ss telomeric binding protein Cdc13p, recruiting telomerase to chromosomal ends in S-phase. Recruitment and activation of telomerase is till now not well understood in human, and the role of EST1A/SMG6 is still uncertain but it might regulate telomerase through TERRA. Because TERRA-mimicking oligonucleotides inhibit telomerase activity in vitro and TERRA is more abundant when telomeres are long [20, 21], telomerase may be regulated by TERRA in a telomere-length dependent manner.

Consistent with this finding, tumour tissues show a lower level in TERRA expression than in corresponding normal tissue. [20] Additionally, tumour-derived and in-vitro-immortalized

cell lines show a low level in TERRA expression and presence of telomerase when compared with cell lines using the ALT mechanism to maintain telomere length. [24] Overexpression of RNaseH in yeast reduces TERRA levels and overcomes the short telomere phenotype, indicating that a DNA/RNA hybrid was responsible for this effect [25]. Another line of evidence, that TERRA plays a role in inhibiting telomerase is the fact that overexpression of TERRA molecules in yeast by using a strong Gal promoter leads to shortening of transcribed telomere. [25]

2.3.4.2 Heterochromatin

Large non-coding RNAs (ncRNAs) can cause changes in epigenetic by recruiting chromatin-remodelling complexes to genomic loci. For example, X chromosome inactivation in females, a large ncRNA, Xist, is involved in inactivating one of two X chromosomes. It would be possible, that TERRA functions as a mediator for recruitment of chromatin-remodelling enzymes at telomeres to establish or maintain the heterochromatic state.
Another similarity between X chromosome inactivation and TERRA is the fact, that both are regulated by SMG proteins. Female mouse embryonic stem cells fail to undergo X-inactivation, when they are depleted for SMG proteins. [26] Leading to the suggestion, that SMG protein might play a role in chromatin organization involving long ncRNAs. TERRA has been shown to colocalize with Xist and specifically with the distal telomere of the inactivated X chromosome in female mouse embryos. [27]
But TERRA localization on the inactive X chromosome does not depend on the expression on Xist, indicating that TERRA is not depending on X chromosome inactivation but plays a role in telomere specific heterochromatinization.

2.3.4.3 TERRA and development

It has been shown that TERRA may be regulated developmentally and therefore be important for manipulating some features of chromosomal transactions occurring during cellular differentiation events. It is interesting to know that TERRA can be found in undifferentiated ES cells and can be found associated with both X chromosomes in females and with X and Y chromosome in males. After differentiation, only one of the two sex chromosomes is marked by TERRA, the inactivated X chromosome in females and the Y chromosome in males, showing that TERRA is developmentally regulated. [27] And also when differentiated fibroblasts are induced to form pluripotent stem cells with reprogramming factors Oct3/4, Sox2, Klf4 and c-myc, TERRA levels are strongly increased [22, 28]. It is not understood if the increase in TERRA level is due to reprogramming event, or if reprogramming factors function as transcriptional regulators of TERRA.

3 Aims of the study

The aims of this study are:

1. Isolation of DNA and RNA from tissue samples of astrocytic brain tumours WHO grade II to IV and cell culture cells, and comparison of different isolation methods.

2. A quantitative Real-time PCR assay will be established for the relative measurement of telomere DNA length normalized to a reference gene, and the validation of this assay with another method, for example terminal restriction fragment (TRF) analysis by Southern blot.

3. The established PCR assay will be validated with ALT positive and ALT negative cell lines if it can distinguish the telomere maintenance mechanism. Cells can use telomerase or a telomerase independent mechanism termed alternative lengthening of telomeres (ALT) [29], resulting defined telomere length characteristics [30].

4. PCR Assays to detect TERRA will be established.

5. TERRA promoter methylation will be analysed and compared with expression levels.

6. The telomere length and expression will be correlated to clinical parameters (survival of patients) and further existing data (telomerase activity, expression levels of telomerase and shelterin genes)

4 Methods and Materials

4.1 Tissue samples, clinical data and cell lines

52 tissue samples were derived form 46 patients suffering from astrocytomas and 6 non-tumour control tissues were acquired from two patients by removal of epileptic foci. The samples were frozen on liquid nitrogen after surgical removal and stored at -80°C and liquid nitrogen, respectively. The astrocytomas were histologically graded as WHO grade II (diffuse astrocytoma, DA), WHO grade III (anaplastic astrocytoma, AA) and WHO grade IV (glioblastoma multiforme, GBM) by Johannes Hainfellner at the Institute of Neuropathology (AKH Vienna).

The recruitment phase of the study was for GBMs (grade IV), AA (grade III) and DA (grade II) astrocytomas from December 2004 to September 2007 for GBMs, and from September 2000 to April 2007 for AA and DA. The clinical data from the patients were obtained from MD Matthias Preusser and MD Christine Marosi (AKH Vienna) and age of patients were calculated (Table 4.1). The local research ethics committee approved the study, and all patients provided written informed consent. Patients were followed up until October 01, 2009. Patient age at diagnosis, at last follow up and survival were calculated from date of their first operation at which grade II to IV disease was confirmed histological.

Patient treatments for low grade astrocytomas were heterogeneous. However, all GBM patients were treated with standard therapy. Standard therapy at the AKH Vienna is surgery with concomitant chemo- and radiotherapy with akylating agents (1995-2000 CCNU or fotemustine/dacarbacine) and since 2000 Temozolamide (personal communication C. Marosi). Exept one case all analysed GBMs originated as primary tumours (GBM12 was a secondary tumor).

Table 4.1 **Number of analysed cases with diagnosis, grading and patient data (gender and age at diagnosis)**

WHO grade		Total	♀	♂	Age at diagnosis
Diagnosis	WHO grade	n			SEM ± SD/year
DA	II	12	8	4	54 ± 19
AA	III	6	3	3	45 ± 15
GBM	IV	28	10	18	46 ± 18

DA = diffuse astrocytomas, AA= anaplastic astrocytomas, GBM= glioblastomas multiforme

14 cell lines were analysed in this study (Table 4.2). 12 of this cell lines are derived from astrocytomas, 1 from a desmoplastic cerebellar medulloblastoma (Daoy)) and 1 from a primary human osteosarcoma (SAOS-2).

Table 4.2 **Cell lines**

Cell line	Medium	Source
CCF-STTG1 (CRL-1718)	RPMI +10% FCS	ATCC
DBTRG/05MG (CRL-2020)	RPMI +10% FCS	ATCC
KG-MH	RPMI +10% FCS	Established from patient material
KM-YH	RPMI +10% FCS	Established from patient material
LN-140	MME + 10% FCS	Dr. Tribolet, Lausanne (Switzerland)
MGC	MME + 10% FCS	Dr. Kurata, Tokyo (Japan)
MR1	MME + 10% FCS	Dr. Kurata, Tokyo (Japan)
T98G (CRL-1690)	MME + 10% FCS	ATCC
U-373 MG (HTB17)	MNP + 10% FCS	ATCC
YT-BO	RPMI +10% FCS	Established from patient material
YU-PM	RPMI +10% FCS	Established from patient material
Hs683 (HTB138)	DMEM + 10% FCS	ATCC
Daoy (HTB168) *	MNP + 10% FCS	ATCC
SAOS-2 (HTB85) **	McCoys + 15% FCS	ATCC

ATCC numbers are given in brackets

* originated form desmoplastic cerebellar medulloblastoma

** originated from primary human osteosarcoma

MNP . . . Minimal essential medium eagle with non essential amino acids and pyruvat

MEME . . . Minimal essential medium eagle

RPMI . . . RPMI-1640 with following additives: adenine, ATP, L-cystine, HEPES

DMEM . . . Dulbecco´s modified Eagles medium

4.2 Biochemical methods

4.2.1 Homogenisation of tissues

Materials:

- Small glass trough
- Big glass trough
- Scalpel
- Forceps
- Spattle
- Ultraturax
- 3x 500ml cups with about 200ml H_2O (for cleaning the Ultraturax)

A big glass trough is filled with ice and a smaller one is placed within the ice. 14ml tubes (Falcon) per analyses, spattle, forceps and tubes with snap-frozen tissue samples are placed on dry ice.
Tissue samples are placed on the ice-cooled glass trough with forceps. Semi-frozen tissues are quickly cutted with a scalpel. The taken sample (~50 to 100µg) should be representative of the whole tumour sample and is transferred quickly with the cooled spattle semi-frozen into the cooled 14ml tubes. The rest of the tissue is frozen back on liquid nitrogen for later analyses.

Analyses for RNA/DNA Isolation with TRIZOL® Reagent (Invitrogen, USA):
Place the tissue samples in the 14ml tubes and keep on dry ice. Before addition of 500 µl of the Trizol Reagent tubes with the tissue samples are kept shortly at room temperature. Tissue samples with Trizol Reagent are homogenized by the power homogenizer Ultra-TURRAX T8 dispersing tool S8N-5G (IKA, Germany). In detail, 3 pulses of max 5 seconds with power level 2 have been applied. The lysat is transferred to 1.5ml tubes with O-ring seal (Eppendorf) and stored at -80°C.

Analyses for Telomerase Activity (Protein-CHAPS-lysats):

1x CHAPS Lysis Buffer:

- 10 mM Tris-Cl (pH 7.5)
- 1 mM EGTA
- 1 mM $MgCl_2$
- 0.1 mM Benzamidine
- 5 mM β-mercaptoethanol
- 0.5% CHAPS
- 10% Glyerol

Prepare 2 ml cryovials (greiner bio-one) for each tissue sample. Chaps lysis buffer is supplemented with RNasIn in the following way: 1,300 µl of Chaps lysis buffer and 6.5 µl of RNasIn (Promega 40/µl) for 6 analyses. Give 200µl of Chaps + RNasIn to each cryovial and put them on ice. Tissue samples are cutted and prepared as described before. Tissue sample is given directly into the cryovial with lysis buffer on ice.

Sample is homogenized by Ultraturax on ice for 1 – 5 seconds (max 2-3 pulses with power level 2), remaining pieces are not homogenized to avoid heating of the sample. Incubate the homogenates for 30 minutes on ice. Transfer the lysats to a fresh tube and centrifuge (1,200g/20minutes/2-8°C). Lysats are than splitted in two aliquots (100µl and 60µl) and frozen on dry ice and stored at -80°C.

From the remaining pellet, a high quality DNA can be isolated [30]. The pellets were stored at -80°C.

Protein extracts can be used for detection of proteins by western blotting. For this purpose, protease inhibitor complete (Roche) and phosphatase inhibitor cocktail phosphatase inhibitors 1mM NaVO4 and 5mM NaF are added to the Chaps lysis buffer to avoid protein degradation.

4.2.2 Isolation of total RNA from Trizol

Materials:

- Chloroform
- Isopropanol
- 75% EtOH
- RNaseInhibitor
- RNase free H_2O

Phase separation:

100µl of Chloroform (per 500µl Trizol) are added to the lysat, vortexed and incubated at room temperature for 2 to 3 minutes.

Then the sample is centrifuged (12,000g/15minutes/2-8°C). After that three phases are visible: the organic phase, the interphase and the RNA-containing aqueous phase.

Precipitation of RNA:

The aqueous phase is given in a fresh tube (there should be no part of interphase). The remaining is left for following DNA and protein isolation and can be stored at -80°C.

To precipitate RNA, 250µl (per 500µl of Trizol) of isopropanol is added to the aqueous phase and mixed by inverting the tube and incubated for 10 to 15 minutes at room temperature. After that, the precipitated RNA is centrifuged (12,000g/10min./2-8°C) and supernatant is removed. RNA pellet should be visible now.

Washing of RNA:

The RNA-pellet is washed with at least 500µl of 75% ethanol (per 500µl of Trizol). The sample is briefly vortexed than centrifuged (7,500g/5minutes/2-8°C). Supernatant is totally removed.

Dissolving of RNA:

Air-dry the RNA-pellet (15-20 min at RT?), but the pellet should not completely dry, because it will not be resolvable very good. To dissolve RNA RNase free H_20 with RNasIn (Promega 40/µl) is prepared (e.g. 200µl of H_20 and 2µl of RNasIn). When the pellet is dry, dissolve it in 30µl of H_2O and RNasIn.

4.2.3 Isolation of genomic DNA from Trizol

Materials:

- 100% Ethanol
- 0.1M Sodium citrate in 10% Ethanol
- 75% Ethanol
- 8mM NaOH
- 0.1M HEPES
- 15mM EDTA

Genomic DNA is isolated from the TRIZOL-lysat after RNA isolation. After completely removing the aqueous phase, the DNA is isolated from the interphase and the organic phase.

Precipitation of DNA:

DNA is precipitated with 100% ethanol. 0.3 ml of ethanol (per 500µl Trizol) are added and mixed by inverting the tube. The sample is incubated at room temperature for 2 to 3 minutes and centrifuged (2,000g/5minutes/2-8°C).

DNA washing:

Supernatant is removed and the DNA pellet is washed two times in a solution of 0.1M sodium citrate in 10% ethanol. 500µl (per 500µl Trizol) are added to the pellet. Wash the pellet 30 minutes at 15 to 30°C by gently shaking (350 rpm) it on a thermomixer. Centrifuge the sample (2,000g/5minutes/2-8°c). Supernatant is removed and the washing step is repeated. Pellet is dissolved in 0.75 – 1 ml (per 500µl Trizol) of 75% ethanol. Wash it 10 to 20 minutes on a thermomixer at 15 to 30°C. Again centrifuge the sample (2,000g/5minutes/2-8°C).

Dissolving of DNA:

Remove supernatant and let the pellet air-dry. When the pellet is dry (20 min at RT)), it is redissolved in 8mM NaOH. Dissolving DNA at low pH is recommended, since DNA can not be redissolved in water or Tris-buffer.

Normally 50µl of 8mM NaOH are used for redissolving DNA. After redissolving the pellet, pH is adjusted with HEPES to 7 – 8 and supplemented with 1 mM EDTA as described by the manufacturer's instructions. Once pH is adjusted, DNA was stored at -20°C or 4°C.

4.2.4 Isolation of DNA with Maxwell® DNA Purification Kit

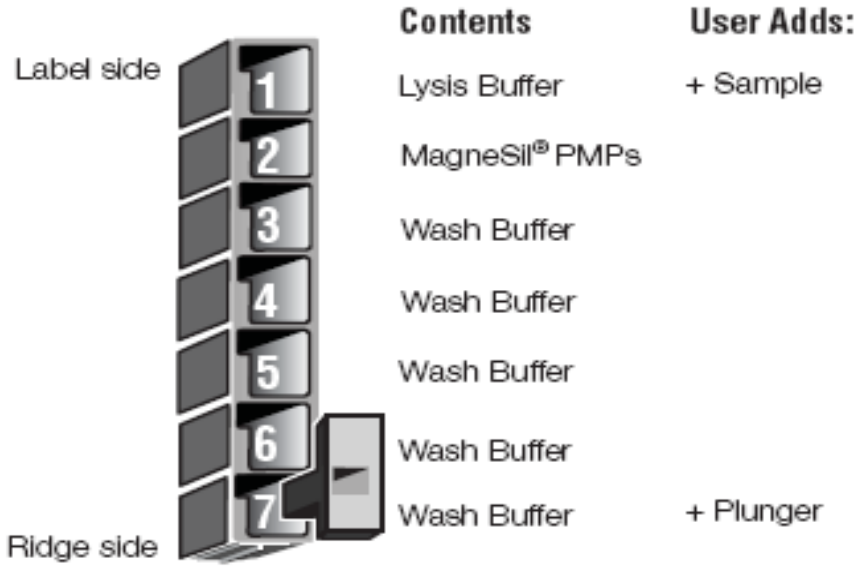


Figure 4.1 **Maxwell 16 DNA Purification Cartridge**. Figure shows the contents of a cartridge of Maxwell 16 Tissue DNA Purification Kit. Figure taken from Manual to Maxwell 16 Tissue DNA purification system (Promega).

Each cartridge (Fig. 4.1) to be used is placed in the holder and opened. A plunger is placed in the last well of each cartridge (well 7). Blue elution tubes are prepared with 300µl of elution buffer.

Fresh or frozen tissue is added to well 1 (containing lysis buffer).

The Maxwell®16 instrument is switched on and "DNA" is chosen from the menu and then press button RUN/STOP. Then choose "Tissue" from the menu and again press RUN/STOP. Instrument shows the chosen menu, check if the right parameters for DNA isolation are chosen, then press RUN/STOP. Open the door of the instrument and by pressing RUN/STOP the platform is extended. Place cartridges and elution tubes within the instrument and press RUN/STOP. When the door is closed, the program starts automatically. At the end, tubes are removed and stored at -20°C or 4°C.

4.2.5 Determination of DNA concentration

4.2.5.1 NanoDrop ND-1000 Spectrophotometer (Peqlab)

Open the program NC-1000 3.3.0 on the PC. A window is opening, and the instrument is demanding for water. Place 1 – 2 µl of water on the instrument and press Ok.

Remove water from instrument and displace it by 1 – 2 µl of 1xTE buffer and press BLANK. Now the samples can be measured. Put 1 – 2 µl of the sample on the instrument and press MEASURE. The instrument gives you the amount of DNA but also the purity (260/280). This is repeated with every sample to be measured. Results are stored in an Excel file.

4.2.5.2 Qubit® Fluorometer (Invitrogen, Fisher Scientific)

Materials:

- Quant-iT™ dsDNA BR reagent
- Quant-iT™ dsDNA BR buffer
- Quant-iT™ dsDNA BR standard#1
- Quant-iT™ dsDNA BR standard#2

All reagents should be brought to room temperature before using them. Prepare one tube for every sample to be measured on Qubit fluorometer and for each standard. For Quant-iT working solution, the dsDNA BR (broad range) reagent is diluted 1:200 with the Quant-iT dsDNA BR buffer. 200 µl of this working solution are needed for every standard and every sample to be measured (Table 4.3).

Table 4.3 **DNA quantification on Qubit fluorometer**

	Standards µl	Samples µl
Volume of Working solution	190	199
Volume of Standard	10	0
Volume of Sample	0	1
Total volume in each assay tube	200	200

For standards: 190 µl of working solution and 10 µl of standard 1 and standard 2, respectively are mixed in an 250 µl tube and are briefly vortexed and spinned down.

For samples: 199 µl of working solution and 1µl of the DNA to be measured are mixed in and 250 µl tube and also briefly vortexed and spinned down. (If necessary, bigger amounts of DNA can be used for measurement. And in the opposite, if DNA is too concentrated, an appropriate dilution of DNA is used for quantification).

All samples and standards are incubated for 2 minutes at room temperature. Qubit Data Logger is opened on the PC and the tubes are placed in the fluorometer. First the instrument is calibrated with standard 1 and standard 2.

Then sample DNAs are quantified by placing them in the instrument. The results of measurement are saved as Excel file and the formula to calculate the amount of DNA is:

Concentration of DNA = QF value × (200)/µl of sample

4.2.5.3 Determination of RNA concentration

Samples are stored at -80°C and are heated for 10 minutes to 55-60°C after every thawing to redissolve RNA and to avoid RNA aggregates.
RNA concentration can be measured with NanoDrop ND-1000 Spectrophotometer or Qubit Flourometer (see also 4.2.5 Determination of DNA concentration).
For determination of RNA concentration with Qubit fluorometer the Quant-iT™ RNA BR Kit is used.

4.2.6 Synthesis of complementary DNA (cDNA)

cDNA- synthesis was done in 20 µl by the manufacturer's protocol for RevertAid First Strand cDNA synthesis kit (Fermentas, Germany). Therefore, RNA sample is thawed, centrifuged and placed on ice. 1µg of RNA were given in a 0.5 ml PCR tube and filled up to a volume of max. 11.5 µl with ddH_2O. A random hexamer primer (5´-N_6-3´) was added to the RNA (1µl of primer; 0.2 µg/µl) and incubated for 5 minutes at 70°C. Afterwards the tube was immediately put on ice. 4µl of a 5x reaction buffer was added and heated for 5 minutes at 25°C. Finally, 1µl of RevertAid M-MuLV reverse transcriptase was added and the total reaction mixture was incubated for 10 minutes at 25°C, 60 min at 42°C, 10 min at 70°C. The reaction was then stopped on ice. The cDNA sample was diluted 1:25 to a final volume of 500µl by adding 480µl of ddH_20. This would allow 50 PCR experiments with a 20 µl scale.

Mastermix (for each sample):

- 1µl Random hexamer primer (0.2µg/µl)
- 4µl 5x Reaction Buffer
- 0.5µl Ribonuclease Inhibitor (20u/µl)
- 2µl dNTP Mix (10mM)
- 1µl M-MuLV Reverse Transcriptase (200 U/µl)

4.3 Polymerase chain reaction (PCR)

PCR is used to amplify short, well defined regions of a DNA. Two oligonucleotide primers, target DNA and DNA polymerase are necessary for the amplification. DNA polymerase catalyzes the replication of the DNA in adding nucleotides to the 3´OH-end of the primer and synthesizing complementary DNA sequences. Oligonucleotide primers have usually a length of 15 – 30 bases, which can vary greatly. The PCR method can be used to amplify specific peaces of a DNA strand, generally 100bp to 3 kb in length.

4.3.1 Steps in PCR

PCR consists of different phases:

1. Denaturation:

In this phase, the DNA double strand is separated into the two single strands at a high temperature (95°C). Primers are now able to bind to the single strands.

2. Annealing

This step is needed for the annealing of primers to the now single stranded DNA. The temperature here is primer-specific.

Exactly one should say, the primers bind anywhere and detach very quickly. But if a primer binds to the correct position, this binding is stable. For annealing, primers have to be in molar excess over DNA, otherwise separated strands of DNA would anneal.

3. Extension

In this phase, polymerase produces the counterpart of the template DNA. This step is carried out at a temperature of 72°C. The following cycle starts again with denaturation. The primers bind in the following annealing phase to the template DNA and to the products of previous cycles.

4.3.2 Primer design and PCR components

4.3.2.1 Primer design

Primers are oligonucleotides and are ordered from VBC Genomics (Vienna). The primer sequences are part of the sense and anti-sense strand of the double stranded DNA to be amplified. So, the primer sequences are specific for each amplified dsDNA and are already be known. The primer sequences in Table 4.4 were taken from literature or designed with the Clone Manager9 program (Scientific & Educational Software, NC, USA). Information on DNA and RNA sequences for genes and subtelomeric regions were obtained from reference sequences, NC and NM accessions, respectively, from public databases at the National Center of Biotechnology Information (NCBI; http.//www.ncbi.nlm.nih.gov). Sequence data were imported into the Clone Manager9 program.

Primer sequences for two housekeeping genes 36B4 and β-globin were from literature [31]. 36B4 codes for the acidic ribosomal phosphoprotein P0 and β-globin bilds together with α-globin the most commonly form of haemoglobin.
Primers for amplifying repetitive telomeric repeats have to be specially designed and were taken from literature [32]:

- They are designed in a way, that the polymerase can start at the 3´-end, but only if primers are bound to telomeric sequence, and not, if two primers bind. If two primers bind, four base pairs and every fifth and sixth base pair give a mismatch. And so, the base on the 3´-end stays unpaired and polymerase can not start elongation, if two primers pair.
- Primers should bind at the end of previous produced amplicons and not somewhere in the center. So these primers have six bases on their 5´-end, which do not pair, when the rest of the primer is annealed to telomeric sequence. The counterpart for this six bases is build on the 3´-end of each produced amplicon and should inhibit binding of primers somewhere in the center of a amplicon.

- The GC-content should be more than 50% and all bases should be distributed equally and melting temperature of the two primers should not differ more than 1°C.

All primers used were analysed for proper function with the CloneManager program (Table 4.5).

Table 4.4 **Primersequences**

a) Telomere length assay

Primer	Length
Tel1b	5´-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GGT-3´
Tel2b	5´-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3´
hbg3	5´-TGT GCT GGC CCA TCA CTT TG-3´
hbg4	5´-ACC AGC CAC CAC TTT CTG ATA GG-3´
36B4u	5´-CAG CAA GTG GGA AGG TGT AAT CC-3´
36B4d	5´-CCC ATT CTA TCA TCA ACG GGT ACA A-3´

Primers tel1b und tel2b were described in [32]

Primers 36B4u und 36B4d were described in [31]

Primers hbg3 und hbg4 were described in [33]

b) 2p and 18p TERRA expression

Primer	Sequence
chr2_590	5´-TAA GCC GAA GCC TAA CTC GTG TC-3´
chr2_738	5´-GTA AAG GCG AAG CAG CAT TCT CC-3´
chr18_617	5´- CCT AAC CCT CAC CCT TCT AAC-3´
chr18_725	5´-ACC AGC CAC CAC TTT CTG ATA GG-3´
2253BACT_for	5´-GGA TGC AGA AGG AGA TCA CTG-3´
2253BACT_rev	5´-CGA TCC ACA CGG AGT ACT TG-3´

Primers were designed with Clone Manager9 program (Scientific & Educational Software, NC, USA) to amplify the subtelomeric region of short arms of chromosome 2 and 18, respectively.

c) Methylation specific PCR

Primer	Sequence
MP_HC2 FWD	5´- GTA TTG TAG GTG TAT AGT TGT ATA AG -3´
MP_HC2 REV	5´- TAA AAT TCC ACC TAT CTC TAT AC -3´
MP_HC2_REV_S	5´- TAA TAC GAC TCA CTA TAG GGG ATC GAT CTA AAA TTC CAC CTA TCT CTA TAC -3´
MP_HC18 FWD	5´- GTA AGG TTT GTG TTG ATT AGG A -3´
MP_HC18 REV	5´- AAA ATA AAA CTA TAT TCT CAT CAA C -3´
MP_HC18_REV_S	5´- TAA TAC GAC TCA CTA TAG GGG GCC GAT CCA AAA TAA AAC TAT ATT CTC ATC AAC -3´

Primers MP_HC2 FWD, MP_HC2 REV, MP_HC18 FWD and MP_HC18 REV are described in [24]

Primers MP_HC2_REV_S and MP_HC18_REV_S are modified (extended at the 5' end by T7 primer sequences)

Table 4.5 **Primer properties**

a) Telomere length assay

Primer	Length (nt)	N-content A-G-C-T	GC-content (%)	Tm (°C)
Tel1b	39	0-18-1-20	48.7%	78.4°C
Tel2b	39	5-3-18-13	53.8%	72,3°C
hbg3	20	2-5-6-7	55%	56,7°C
hbg4	23	6-4-8-5	52.5%	56,1°C
36B4u	23	7-8-4-4	52.2%	56,5°C
36B4d	23	8-3-8-6	44%	57,9°C

b) 2p and 18p TERRA expression

Primer	Length (nt)	N-content A-G-C-T	GC-content (%)	Tm (°C)
chr2_590	23	6-7-5-5	52%	66°C
chr2_738	23	7-6-6-4	52%	66°C
chr18_617	21	5-11-0-5	52%	61°C
chr18_725	23	7-6-6-4	52%	66°C

c) Methylation specific PCR

Primer	Length (nt)	N-content A-G-C-T	GC-content (%)	Tm (°C)
MP_HC2 FWD	26	7-0-8-11	30	56
MP_HC2 REV	23	8-7-0-8	30	54
MP_HC2_REV_S	51	17-13-6-15	37	71
MP_HC18 FWD	22	5-0-8-9	36	58
MP_HC18 REV	25	13-5-0-7	20	53
MP_HC18_REV_S	54	21-13-7-13	37	73

Information about primer properties are taken from CloneManager 9 program, primer details and from VBC genomics synthesis reports.

Dissolving of delivered primers:

- Briefly spin down lyophilized primers
- Dissolve primer in given volume of 1xTE (synthesis report) for 100pmol/µl
- Vortex and briefly spin down
- Dissolve primers 10 minutes at 37°C
- Again, vortex and briefly spin down
- Put primers on ice
- Prepare a 1:50 working solution of primers in 0.1x TE (e.g. 10µl of forward primer, 10µl of reverse primer and 480µl of 0.1x TE)

4.3.2.2 PCR components

PCR components for all primers used were the same (tel1b+tel2b, 36B4d+36B4u, hbg3+hbg4, chr2_590+chr2_738, chr18_617+chr18_725)

10 µl 2 x PCR Master Mix (Promega, Promega, M7502)
1 µl Primer forward and reverse (2pmol/µl)
8 µl (20ng) template DNA
20 µl total volume

4.3.2.3 PCR protocol

A PCR was performed for amplification of telomeric repeats with primers tel1b and tel2b, for the single copy genes 36B4 and ß-globin with primers 36B4u and 36B4d and primers hbg3 and hbg4. For specific amplification of TERRA from chromosomal end 2p and 18p primers chr2_590 and chr2_738 and primers chr18_617 and chr18_725 were used on a thermocycler HYBAID Omn-E (GenXpress) under the following PCR conditions (Table 4.6).

Table 4.6 **PCR conditions**

PCR phase	Temperature °C	Time	Number of cycles
Initial denaturation	95	2 minutes	1
Denaturation	95	15 seconds	28
Annealing/extendsion	60	1 minute	28
Final extension	60	5 minutes	1

Cycling conditions are the same for primers tel1b + tel2b, 36B4d + 36B4u and hbg3 + hbg4

4.4 Polyacrylamidegelelectrophoresis (PAGE)

Materials:

Gels

- 12.6 ml ddH2O
- 375 µl 40 x TAE
- 1.95 ml Acrylamide/Bis 19:1 Solution (Biorad)

for polymerisation start add and mix:

- 20.8µl 25% APS (Ammoniumperoxodisulfate, MERCK, Germany)
- 20.8µl TEMED (N,N,N´,N´-Tetramethylethylenediamine, Sigma, USA)

poor in prepared trays for gels (BioRad PAGE System3)

40 x TAE

- 96.8 g Tris base
- 22.8 ml glacial acetic acid
- 40ml EDTA (pH 8; 0.5 M) and fill to 1000 ml with ddH2O and autoclave

4.4.1 Preparing the samples:

3µl aliquots of PCR products are added to 1.5ml or 0.5ml tubes (Eppendorf) containing each 7µl ddH2O and 2 µl of 6 x loading dye. 2µl of a 50bp molecular weight marker (Fermentas, #SM0373) are mixed in a tube with 8µl of ddH2O and 2 µl of 6x loading dye.

4.4.2 Running the gel:

Gel electrophoresis was performed at constant 80V for 90 minutes or until the dye reaches the end of the gel with a power supply (Power Pac 3.000, Biorad, USA).

4.4.3 Visualisation of DNA:

DNA bands in the gel are stained with a 1:10,000 dilution of Vistra Green (Amersham Life Science) and visualised in a Fluorimager 595 (Molecular Dynamics) using the filter 530DF30. Alternatively, gels are stained by EtBr (1:10.000) and visualised with Fluorimager 595 or GelDoc System (BioRad).

4.5 Cloning of PCR products

For direct insertion of Taq polymerase-amplified PCR products in a plasmid vector, TOPO TA Cloning Kit (Invitrogen) was used and the cloning was performed according to manufacturer's protocol. Taq polymerase adds a single 3´-A nucleotide overhang to each end of the PCR products. The PCR products were 75bp for 36B4 and 73bp for hbg. The cloning vector was pCRII-TOPO and contains covalently bound topoisomeraseI, which enabled within minutes direct ligation of the PCR products into the vector.

4.5.1 Preparing competent E.coli cells (Simple and efficient methode - SEM protocol)

Materials:

- TB-Buffer: 10mM Pipes
  15mM $CaCl_2$
  250mM KCl

Adjust the pH to 6.7 with KOH and autoclave

- $MnCl_2$-Buffer: 0.544g $MnCl_2$ (550 mM)
  5ml ddH2O

E.coli TOP10 cells were obtained from strike out plates from old competent cells or from the frozen bacterial LB-stock collection at -80°C. 50µl of over-night culture of E.coli cells are inoculated into 200 ml of LB medium and incubated at room temperature overnight with shaking at 250 rpm. When cell suspension reaches an OD_{600} of ~0.6, the cells are immediately put on ice, and are splitted into 50 ml tubes (Falcons) and incubated for 10 minutes on ice. Solution is centrifuged at 4°C/2,500g for 10 minutes and the supernatant (should be clear, if not, centrifuge for another 10 minutes) is discarded. In the meanwhile, 4 ml of 550 mM $MnCl_2$ solution is added to 36ml of TB buffer. Pellet is resuspended in 40 ml of ice-cold TB buffer (+ $MnCl_2$) and incubated for 10 minutes on ice. Cell suspension is then again centrifuged at 4°C/2,500g for 10 minutes and the supernatant is discarded.
Pellet is resuspended in 10 µl of ice-cold TB buffer and DMSO is added to an end concentration of 7 percent of volume (700µl/10 ml) and incubated for 10 minutes on ice. Finally, the competent cells are aliquoted in 500µl in 1.5ml tubes (Eppendorf), snap-frozen on liquid nitrogen and stored at -80°C.

4.5.2 Cloning reaction

Salt Solution (TOPO TA Cloning Kit, Invitrogen, #K4600-01)

- 1.2M NaCl
- 0.06M $MgCl_2$

pCRII-TOPO (TOPO TA Cloning Kit, Invitrogen)

- 10ng/µl plasmid DNA in:
- 50% glycerol
- 50mM Tris-HCl, pH=7.4
- 1mM EDTA
- 1mM DTT
- 0.1% Triton X-100
- 100µg/ml BSA
- Phenol red

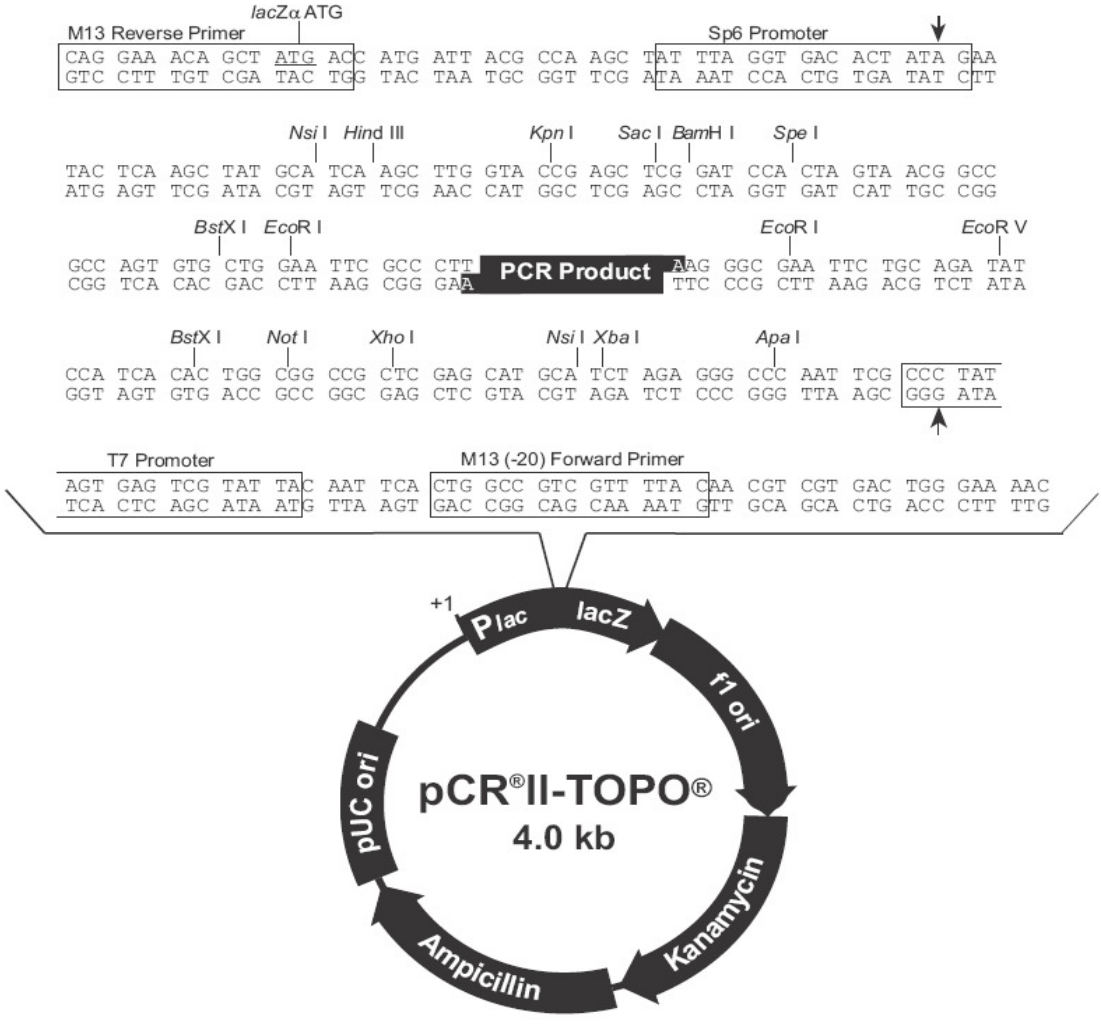


Figure 4.2 **pCRII-TOPO map** Map shows features of the vector and the sequence surrounding the cloning site. Arrows indicate transcription start sites for Sp6 and T7 polymerase. Figure from TOPO TA Cloning Manual, Invitrogen.

The reaction mixture is set-up in a 1.5 ml tube (Eppendorf):

- 2µl of PCR-product (e.g. 36B4 or hbg amplicons)
- 0.5 µl of cloning vector (pCRII-TOPO)
- 0.5 µl of salt solution
- 3µl total volume

Reaction mixture was mixed gently and incubated for 20 minutes at room temperature. After incubation the tube was placed on ice.

4.5.3 SEM Transformation of E.coli cells

Materials

SOB-Medium (for 1 litre):

- 20g Trypton
- 5g Yeast extract
- 0.5g NaCl
- 10ml 250mM KCl ((1.86g NaCl/100ml H_2O)
- pH is adjusted to 7 and autoclaved

SOC-Medium:

- 6ml SOB-Medium
- 60µl (1/100 Vol) 1M $MgCl_2$ solution (9.5g $MgCl_2$/100ml)
- 60µl (1/100 Vol) 2M glucose solution (36g/100ml)

LB-Medium:

- 8g LB
- filled to 400 ml with ddH2O
- autoclaved and when cooled down,
- optional: 400µl of ampicillin (1:1000) stock solution (100mg/ml) are added

LB-Medium für Platten:

- 8g LB
- 6g Agar
- fill to 400 ml ddH2O and autoclave
- optional for Amp100+ plates, 400µl of ampicillin stock solution are added (to an end concentration of 100µg/ml)

SEM (simple and efficient method) chemical transformation was performed to transfer the vector construct into competent E.coli. Competent cells were thawed on ice. The TOPO TA Cloning Kit allows a blue/white screening without IPTG in E.coli TOP10 cells. 1µl of the cloning reaction mixture or 1µl of 10pg/µl pUC vector (for transformation control) are placed in a tube on ice. 200µl of bacteria solution is added and these mixtures are incubated for 20 minutes on ice.

After incubation E.coli cells are heat shocked for 30 seconds at 42°C in water bath. 800? µl of pre-warmed SOC medium are immediately added and the whole mixture is transferred to a 14 ml tube (Falcon). Cells are incubated at 37°C for one hour, by shaking at 230 rpm. In the meantime, 40µl of of an X-Gal stock solution (100 nmol/µl) are spreaded on the ampicillin containing plates. After one hour, the bacteria solutions are transferred to 1.5 ml tubes. The tubes are centrifuged for 3 minutes at 6,000 rpm at room temperature. Supernatant is removed to a small rest of 50-150 µl and the cell pellet is resuspended in this small rest of supernatant. The resuspensions are spread on LB plates with ampicillin and X-Gal and incubated over-night at 37°C.

Next day, white colonies are transferred with a sterile tooth pick to a 14 ml tube (Falcon) containing 5 ml of LB medium with ampicillin and incubated over night at 37°C to obtain cultures of individual clones for further analyses.

4.5.4 Transformation rate

To calculate the transformation rate it is essential to know the amount of pUC19, used in the transformation control reaction. 1µl of pUC19 are used in the control reaction, and 1 µl correspond to 10 pg of plasmid DNA. This is important to know to calculate the transformation rate in number of colonies per µg plasmid DNA transformed.

1µl of pUC19 … 10pg
10 pg . . . x colonies
1 µg . . . x * 10^5 colonies

4.6 Isolation of plasmid DNA

4.6.1 WIZARD Plus SV Miniprep DNA Purification System

Plasmids were isolated from the E.coli cells with the WIZARD Plus SV Miniprep DNA purification system (Promega) with manufacturers' instructions.

Material:

- Cell Resuspension Solution
- Cell lysis Solution
- Neutralization Solution
- Alkaline Protease
- Column Wash Solution
- Spin columns
- Collection tubes

4.6.1.1 Harvesting of E.coli cells

1.5 ml of bacteria culture is centrifuged in 1.5ml tubes (Eppendorf) for 2 minutes at 10,000 g and the supernatant is discarded. The centrifugation step was repeated (2-3x) to increase the amount of bacteria up to 4.5ml culture volume.

4.6.1.2 Lysis and precipitation

The cell pellet is resuspended in 250 µl of resuspension solution and 250 µl of cell lysis solution are added, vortexed and incubated for 3 minutes. After incubation 10µl alkaline protease solution are added, vortexed and incubated for 5 minutes. After 5 minutes, 350 µl of neutralization solution are added and centrifuged for 10 minutes at 14,000 g.

4.6.1.3 Purification and Elution

The supernatant is transferred after centrifugation to a spin column and centrifuged for 1 minute at 14,000 g, flow-through is discarded and 750 µl of column wash solution is added and again centrifuged for 1 minute at 14,000 g and the flow-through is discarded, again centrifuged for 1 minute and flow-through is discarded. Now the spin column is centrifuged for 2 minutes at 14,000 g. 50 µl of nuclease-free water or 1xTE (keeps DNA more stable after multiple freeze/thaw cycles) are placed on the membrane of the spin column and incubated for 2 minutes. To elute the plasmid DNA, spin column is centrifuged for 1 minute at 14,000g. Finally, the spin column is discarded and plasmid DNA is mixed and stored at -20°C.

4.6.2 STET Boiling Plasmid Miniprep (from Stratagene)

Materials:

STET-buffer:

- 8% Saccharose
- 0.5% Triton X-100
- 50mM Tris pH8
- 50mM EDTA

Fill to 1l with ddH_2O and autoclave, stored at +4°C

Lysozyme: 50mg/ml stock in STET buffer, stored at -20°C

RNase A: 100mg/ml stock (in 10mM Tris-HCl pH7.5 and 15mM NaCl heat to 100°C and cool it to room temperature, stored at -20°C

Isopropanol

EtOH 75% (e.g. 7.5ml EtOH and 2.5 ml Water)

Fill cell suspension in 1.5ml tubes (Eppendorf) and centrifuge for 3 minutes at 10,000 g and discard supernatant. The centrifugation step can be repeated (2-3x) to increase the amount of bacteria and the yield of plasmid DNA. For each experiment 100µl STET buffer and 10µl Lysozyme stock are mixed in a 1.5ml tube. 110µl of this STETL buffer are added to each bacteria pellet and resuspended by multiple pipetting.

Suspensions are heated to 95°C for 1 minute. After heating, the suspensions are centrifuged for 10 minutes at 4°C/10,000 g. Pellet is carefully discarded with a toothpeak prior to that soaked with RNaseA solution. Then 110µl of isopropanol are added to the supernatant, mixed and centrifuged for 15 minutes at 4°C/10,000 g. Supernatant is discarded and the pellet is dried for 15 min at RT. The dried pellet is resuspended in 50µl 1xTE.

4.6.3 Alkaline Lysis Miniprep

Materials:

Solution I:

- 15 mM Tris-HCl, pH8, 10mM EDTA, 100µg/ml RNase A; filter-strerilize and store at +4°C

Solution II:

- 0.2N NaOH, 1% SDS; filter-sterilize

Kalium-Acetat, pH5.5 autoclaved and stored at 4°C

Isopropanol

75% Ethanol

Cell suspension is centrifuged in 1.5ml tubes (Eppendorf) for 1 minute at 14,000g, the supernatant is discarded and the pellet is resuspended in 0.3 ml solution I. 0.3 ml solution II are added and the mixture is incubated for 5 minutes at room temperature (RT). After the incubation, 0.3 ml Kalium-Acetat is added slowly and the reaction mix is placed on ice for 5 to 10 minutes, and centrifuged for 10 minutes at 14,000 g. Supernatant is transferred to a new 1.5ml tube with 800µl of isopropanol. Tube is inverted a few times and incubated on ice for 5 to 10 minutes. The suspension is centrifuged for 15 minutes at 14,000 g at RT. Supernatant is discarded and the pellet is washed with 0.5 ml of 75% EtOH. The washed pellet is centrifuged for 5 minutes at RT and 14,000g. Supernatant is discarded and the pellet dried for 5 to 10 minutes. The dry pellet is resuspended in 40µl of 1xTE and stored at -20°C.

4.7 Real-time PCR

The Real time PCR system is based on the detection and quantification of a fluorescent reporter. By recording the amount of fluorescence emission at each cycle, the PCR reaction can be monitored during the exponential phase. At this phase the first significant increase in the amount of the amplicon correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

4.7.1 Method of quantification

One method of quantification is a double-stranded (ds) DNA binding dye chemistry, which quantitates the amount of PCR products (including non-specific amplification products such as primer-dimers) by using a non-sequence specific fluorescent intercalating agent like SYBR Green. SYBR Green binds to the minor groove of dsDNA, but does not bind to single-stranded (ss) DNA (e.g. primers). It shows very little fluorescence as long as it is in solution but shows strong fluorescence after binding to dsDNA, leading to the generation of an excitation-emission profile, which allows the general detection of amplicon accumulation during PCR. At the end of the PCR the products can be heated (e.g. from 60 to 95 °C) and melting curves are generated. Each product does have a characteristic melting temperature. The melting curves can be used to differentiate between specific and non-specific amplicons based on the observed melting temperature (Tm) of the reaction end-products. Additionally, PCR products can be analysed by PAGE.

4.7.2 Real time qPCR assay for relative telomere length

4.7.2.1 PCR components

PCR components for the determination of telomere length measured on 7000 Real-time PCR system (Applied Biosystems):

- 9 µl of template DNA (20 ng)
- 1 µl of forward and reverse primer Tel1b and Tel2b (2pmol/µl each)
- 10 µl of Power SYBR Green Master Mix (ABI)
  20 µl total volume

PCR components for the quantification of expression of telomeres (overall TERRA) on 7500 Fast Real-Time PCR system (Applied Biosystems):

- 4 µl of cDNA
- 0.16 µl of forward and reverse primers Tel1b and Tel2b (10pmol/µl each)
- 3.84 µl of Fast SYBR Green Master Mix (ABI)
  8 µl total volume

PCR components for the quantification of expression of specific TERRA (2p and 18p TERRA) on 7500 Fast Real-Time PCR system (Applied Biosystems):

- 4 µl of cDNA
- 0,16 µl of forward and reverse primers (10pmol/µl each)
- 3.84 µl of Maxima SYBR Green qPCR Master Mix (Fermentas)

  8 µl total volume

4.7.2.2 PCR protocol

The genomic DNA isolated from Trizol (chapter 4.2.3) serves as template for the quantitative real-time PCR assay to measure relative telomere length as T/S ratio. As endogenous control the single (S) copy gene 36B4 PCR assay was used to normalize the telomere (T) PCR assay.

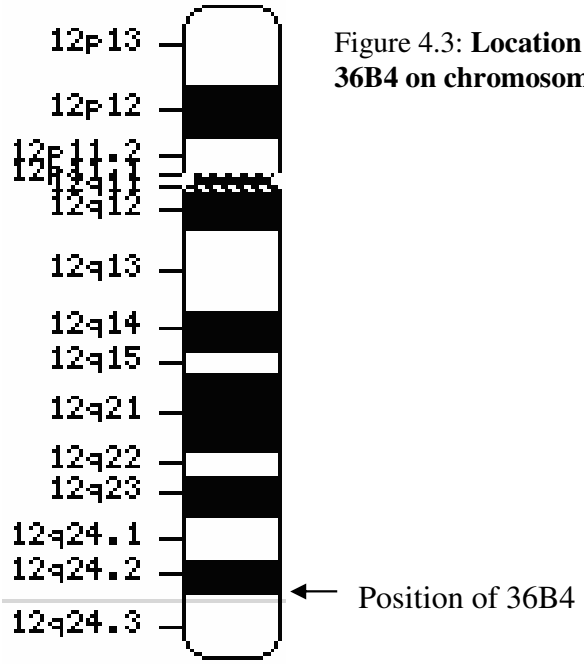


Figure 4.3: **Location of gene 36B4 on chromosome 12**

Relative telomere length was calculated by division of the values obtained for telomeres (T) by the values obtained for 36B4 (S). Real time PCR was performed on ABI PRISM7000 Sequence Detection System (Applied Biosystems).
Reactions were measured in triplicates with each 100nM telomere-specific (T) primers Tel1b and Tel2b or in duplicates with each 100nM single copy 36B4 gene (S) primers 36B4u and 36B4d. DNA samples, primers and PCR Master Mix (Power SYBR Green PCR Master Mix, ABI) were thawed, mixed, down-spinned and placed on ice.

Three thermal cycling parameters were tested for both T and S assay (telomeric repeats and single copy gene 36B4).

60°C:	Initial denaturation:	95°C	10 minutes	1 cycle
	Denaturation:	95°C	15 seconds	
	Annealing and Extension	60°C	1 minute	40 cycles

as described in [32] for both assays.

58°C:	Initial denaturation:	95°C	10 minutes	1 cycle
	Denaturation:	95°C	15 seconds	
	Annealing and Extension	58°C	1 minute	40 cycles

as described in [31] for amplification of gene 36B4.

54°C:	Initial denaturation	95°C	10 minutes	1 cycle
	Denaturation:	95°C	12 seconds	
	Annealing and Extension	54°C	2 minutes	40 cycles

as described in [31] for amplification of telomeric repeats.

Ct values of technical replicates with a standard deviation of Ct >0.5 were excluded from the analysis. Relative quantities (RQ) of T/S gene levels were calculated according to the Pfaffl method [34]. The efficiency (E) of Tel and 36B4 PCR was 0.81 and 0.97, respectively, as determined by standard curves of a DNA probe 1:1 dilution series with 5 dilutions.

Real-time PCR quantitates the initial amount of template and is an alternative to other forms of quantitative PCRs that detect the amount of final amplified product at the endpoint. Real time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each cycle of the PCR in opposite to the endpoint detection.

4.7.2.3 Relative Quantification

Relative quantification calculates the difference in the amount of gene of interest in a sample compared to a reference sample ΔCt is Ct (sample) – Ct (reference sample) for all genes including the reference gene. In the next step the difference between ΔCt for gene of interest and ΔCt for housekeeping gene is calculated (ΔΔCt). Results are expressed as the ratio of amount of gene (expression of gene) in sample of interest versus reference sample and are termed fold change (FC) or relative quantity (RQ). FC or RQ = $2^{-\Delta\Delta Ct}$. For example, the relative amount or expression level of a gene is 3 times higher in the sample of interest compared to the reference sample and compared to the housekeeping gene.

a) Pfaffl Model (Pfaffl, 2001)[34]

$$RQ = (E_{target} + 1)^{\Delta Ct\ target\ (control-sample)} / (E_{ref} + 1)^{\Delta Ct\ reference\ (control-sample)}$$

In this equation, the denominator contains the house keeping gene and the numerator contains the target gene (gene of interest). The formula is corrected for the efficiency (E) of the amplification of the target as well as the house keeping gene. If the efficiency of an assay is 100%, the amount of amplicon doubles with every cycle.

After determination of efficiency of an assay, efficiency + 1 give the increase in amplicon per cycle. What means, when the Ct values of reference and sample are 3 cycles apart, they differ 2^3 = 8fold in the amount of amplicon. If the efficiency is for example 90%, there is a 1.9 fold increase per cycle.

b) ΔΔCt method (Livak and Schmittgen, 2001)[35]

$$RQ = 2^{-(\Delta Ct\ sample - \Delta Ct\ control)} = 2^{-\Delta\Delta Ct}$$

This method is a shortened version of the "Pfaffl" method. Two presumptions are made for this equation:

1. Efficiency of amplification of target and house keeping gene is close to 100%.

2. The difference in expression of the house keeping gene between reference sample and sample of interest in all measured probes is 0. Practically this values should not vary more than 10%

We used the Pfaffl method with measured Efficiencies of the Real time PCR assays in this study.

4.7.2.4 Determination of the Efficiency of a PCR assay

A standard curve is created to determine the efficiency of a PCR assay. Therefore, 1:1 dilution series are used. The Ct-values of each step in the series should be exactly one cycle apart, if the amplification works with an efficiency of 100%. The plot of Ct versus log of initial template concentration should have a gradient close to -3.33. The gradient is negative, because the Ct-value gets smaller with higher template concentration, and should lie between -3.1 and 3.6.

In this study, standard curves were created for telomere repeats (T) and housekeeping gene 36B4 (S) assays. Plasmid pSP73 was used as a template for the standard curve of the T assay, contains an 800 bp fragment of cloned telomere with ~120 telomeric repeats (Fig.4.3). Plasmid pSP73 was kindly provided by Dr. Kiyozuka Y [36] under allowance of Dr. Titia De Lange [37]. Plasmid pCRII TOPO with a cloned PCR fragment of gene 36B4 was used for the standard curve and determination of efficiency for gene 36B4(S) assay.

DNA used for these standard curves had an initial concentration of 20 ng (20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng). Each step was run as a triplicate.

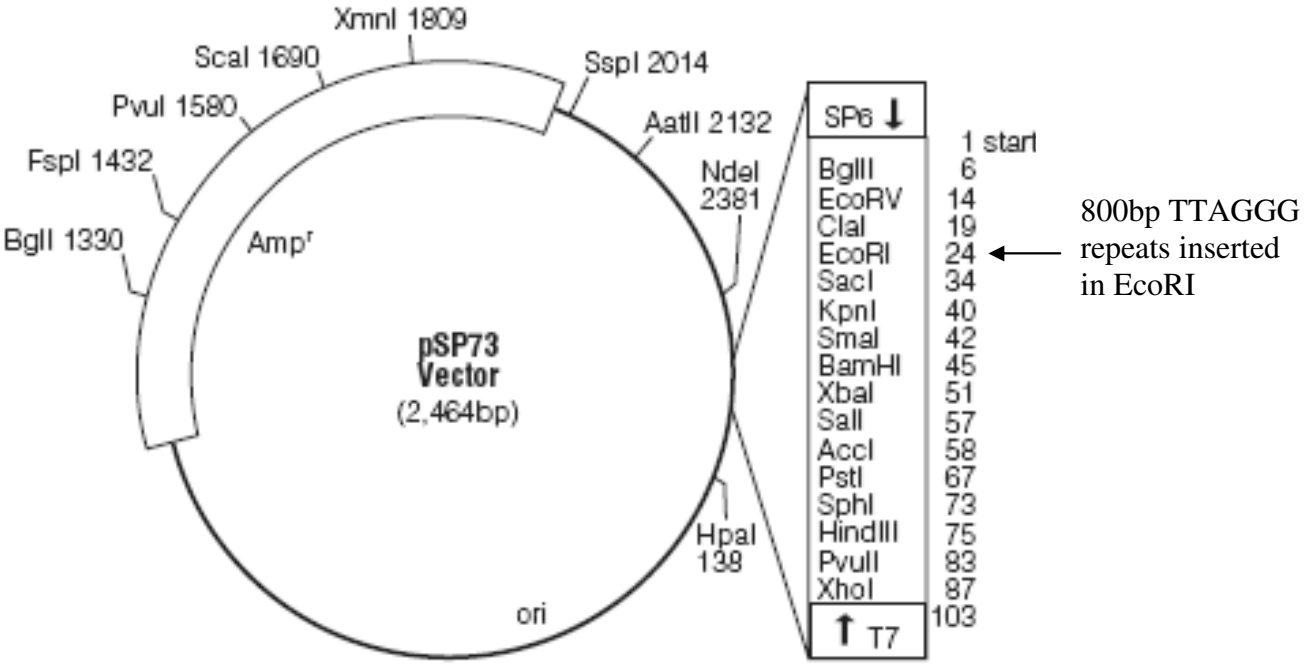


Figure 4.4 **Vector map for pSP73.** The telomere-repeat fragment is cloned within the multi-cloning site between SP6 and T7 primer sites. Insert is cloned into EcoRI cutting sequence.

4.7.2.5 TERRA expression

Overall telomere expression analyses were performed with a two step RT-PCR procedure. First, 1µg total RNA was reverse transcribed with random hexanucleotide primers and RevertAid First Strand cDNA Synthesis Kit (Fermentas), according to manufacturer's protocol. Second, real time PCR was performed on ABI PRISM 7500Fast Sequence Detection System.

For overall TERRA, FAST SYBR Green Master Mix (Applied Biosystems) and Fast program was used (7500 Fast System Software, version 1.4, Applied Biosystems). 40ng total RNA aliquots were analysed with 200nM primer concentration each under conditions as described in Table 4.7 for relative telomere length measurement (Chapter 4.7.2). Relative quantities (RQ) of overall TERRA were calculated as described before.

Table 4.7 **PCR conditions for overall TERRA measurement**

PCR phase	Temperature °C	Time	Number of cycles
Initial denaturation	95	20 seconds	1
Denaturation	95	3 seconds	40
Annealing/extendsion	60	30 seconds	40
Melting curve	95	15 seconds	2
	60	1minute	2

Real time PCR gene expression assays for TERRA quantification specific for chromosome 2p and chromosome 18p were established (Table 4.8). Reactions were performed in triplicates with 200 nM chromosome specific primers from the subtelomeric region chr2_F and chr2_R; or for 18p-TERRA with 200 nM chr18_F and 200 nM chr18_R. Primers are described in table 4.4 and table 4.5. As house keeping gene, again 36B4 was used.

Additionally for the 2p- and 18p-TERRA assays, a second house keeping gene, beta actin, was used to normalize. Beta actin is used often in gene expression studies analysing tissue samples. Primers and concentrations used for beta actin assay: 200 nM 2253BACT_for and 200 nM 2253BACT_rev (Table 4.4). Relative quantities (RQ) of chromosome-specific TERRA were calculated relative to 36B4 and beta actin according to the Pfaffl method [34].

Table 4.8 **PCR conditions for 2p TERRA and 18p TERRA measurement**

PCR phase	Temperature °C	Time	Number of cycles
Initial denaturation	95	10 minutes	1
Denaturation	95	15 seconds	40
Annealing/extendsion	60	1 minute	40
Melting curve	95	15 seconds	2
	60	1minute	2

4.8 Absolute telomere length (TRF Assay)

4.8.1 Preparation of DIG labelled probe for housekeeping gene 36B4

For hybridization-probe preparation pCR II TOPO vector with cloned PCR fragment of 36B4 was used.
20 pg of plasmid DNA (0.2µg 1:10,000 diluted) were used as template for PCR with a PCR DIG Labeling Mix (Roche).

10 µl 10 x Hot Start PCR buffer (Fermentas)
10 µl DIG Labelling Mix (Böhringer-Mannheim, 1 558 706)
2 µl forward and reverse primer (10pmol)
1µl Hot Start Taq DNA polymerase (Fermentas)
6 µl $MgCl_2$ (Fermentas)
11.2 µl (20pg) template DNA
59.8 µl ddH_2O
100 µl total volume

PCR conditions used as described in table 4.6 for primers 36B4d and 36B4u.

4.8.2 Terminal restriction fragment length (TRF) analyses

Telomere restriction fragment (TRF) analyses were done with Telo*TAGGG* Telomere Length Assay (Roche) according to the manufacturer's instructions (Fig.4.4).

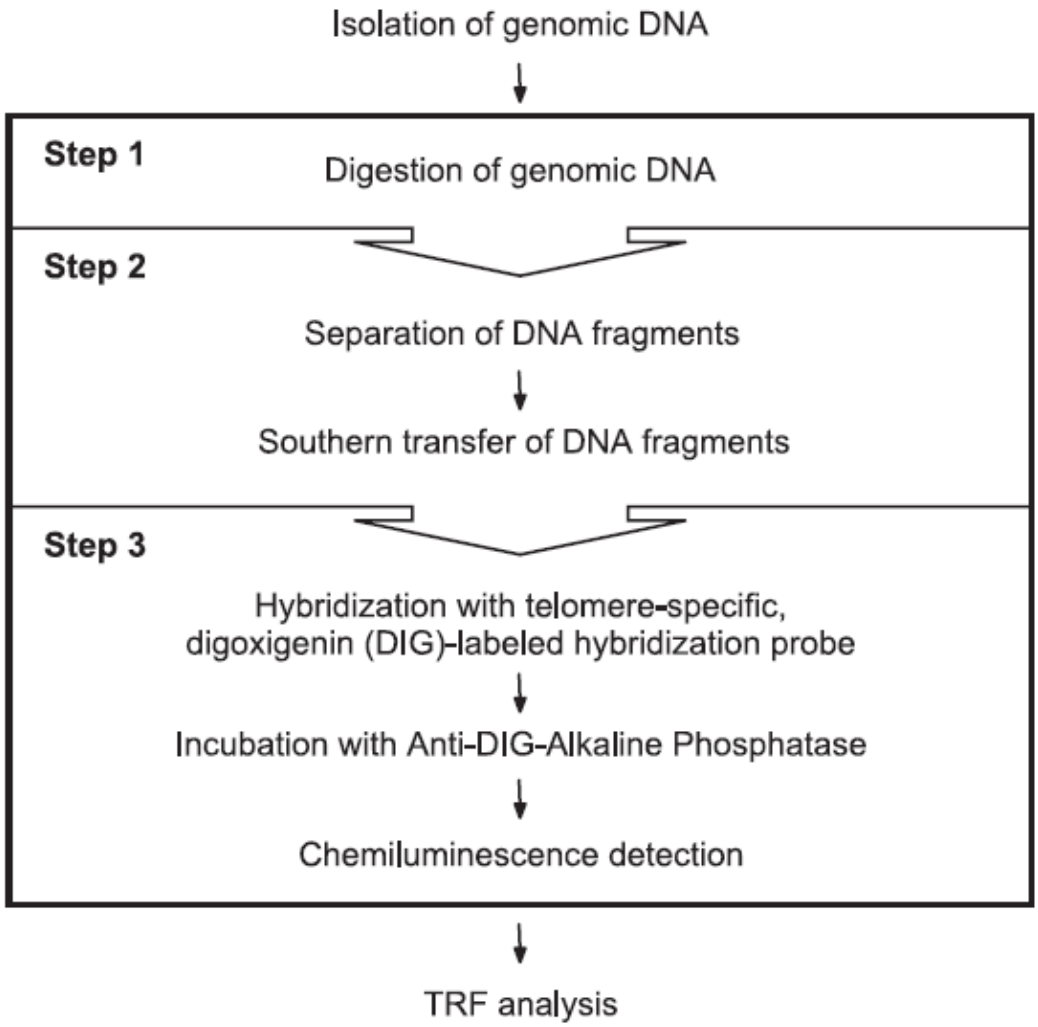


Figure 4.4. **Steps of TRF analysis** (from the manual *T*elo*TAGGG* Telomere Length Assay, Roche)

Materials

20% SDS:

- 100g Sodiumdodecylsulfat
- Fill with ddH_20 to 500ml
- Dissolve by heating (~ 68°C)
- pH 7.2, if necessary adjust pH with a few drops of HCl

20x SSC:

- 175.3g NaCl
- 88.2g sodium citrate
- Dissolve in 800ml ddH_2O
- pH 7.4, If necessary adjust pH with 10N NaOH to 7.4
- Fill with ddH_2O to 1,000 ml

50x TAE:

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100ml 0.5M EDTA pH =8
- Fill to 1,000 ml with ddH_2O and autoclave

10x TAE:

- dilute 50x TAE 1:5 with ddH_2O

Denaturation solution:

- 43.83g NaCl
- 10g NaOH
- Fill to 500ml with ddH_2O

Neutralisation solution:

- 87.66g NaCl
- 30.25g Tris base
- Fill to 500ml with ddH_2O
- Adjust pH to 7.5 with HCl

Sufficient stringent washing buffer I:

- 100ml 2xSSC
- 0.5ml 2%SDS

Sufficient stringent washing buffer II:

- 10ml 2xSSC
- 90ml H_2O bidest
- 0.5ml 2%SDS

4.8.2.1 Digestion of DNA to analyse telomere length

DNA was isolated from tissue samples and cell lines with the Maxwell System (Promega) as described in chapter 4.2.4. Furthermore, control DNAs for long and short telomeres from the *T*elo*TAGGG* Telomere Length assay (Roche) were available. 1µg of DNA was digested with HinfI and RsaI (each 20U/µl) for two hours at 37°C. These restriction enzymes cut the whole genomic DNA, except the telomeres and subtelomeric regions.

4.8.2.2 Gel electrophoresis of digested DNA

The digested DNA and the DIG-labeled DNA-marker were loaded on a 1% agarose gel (Seakem LE Agarose, Cambrex, USA) and run in 1x TAE for ~90 minutes at constant 90 Volt until the blue dye marker reaches the last third of the gel. After electrophoresis, the gel was stained for 15 minutes with Ethidium Bromide Solution (0.25µg/ml) and then washed with

ddH_2O for about 10 minutes. Restriction efficiency was validated by EtBr DNA staining and DNA was visualized with FluorImager595 (Molecular Dynamics).

4.8.2.3 Blotting

The gel was incubated for 10 minutes with 0.25M HCl (till the colour of the dye turns to yellow) and washed with ddH_2O. The gel was then incubated two times for 15 minutes with denaturation solution and again washed with ddH_2O. Now, the gel is incubated for two times 15 minutes with neutralisation solution (colour of dye turns back to blue). In the meantime, the blotting equipment is constructed according to Amersham Bio Sciences manual for capillary blotting (Fig.4.5). The gel is transferred on membrane over night.

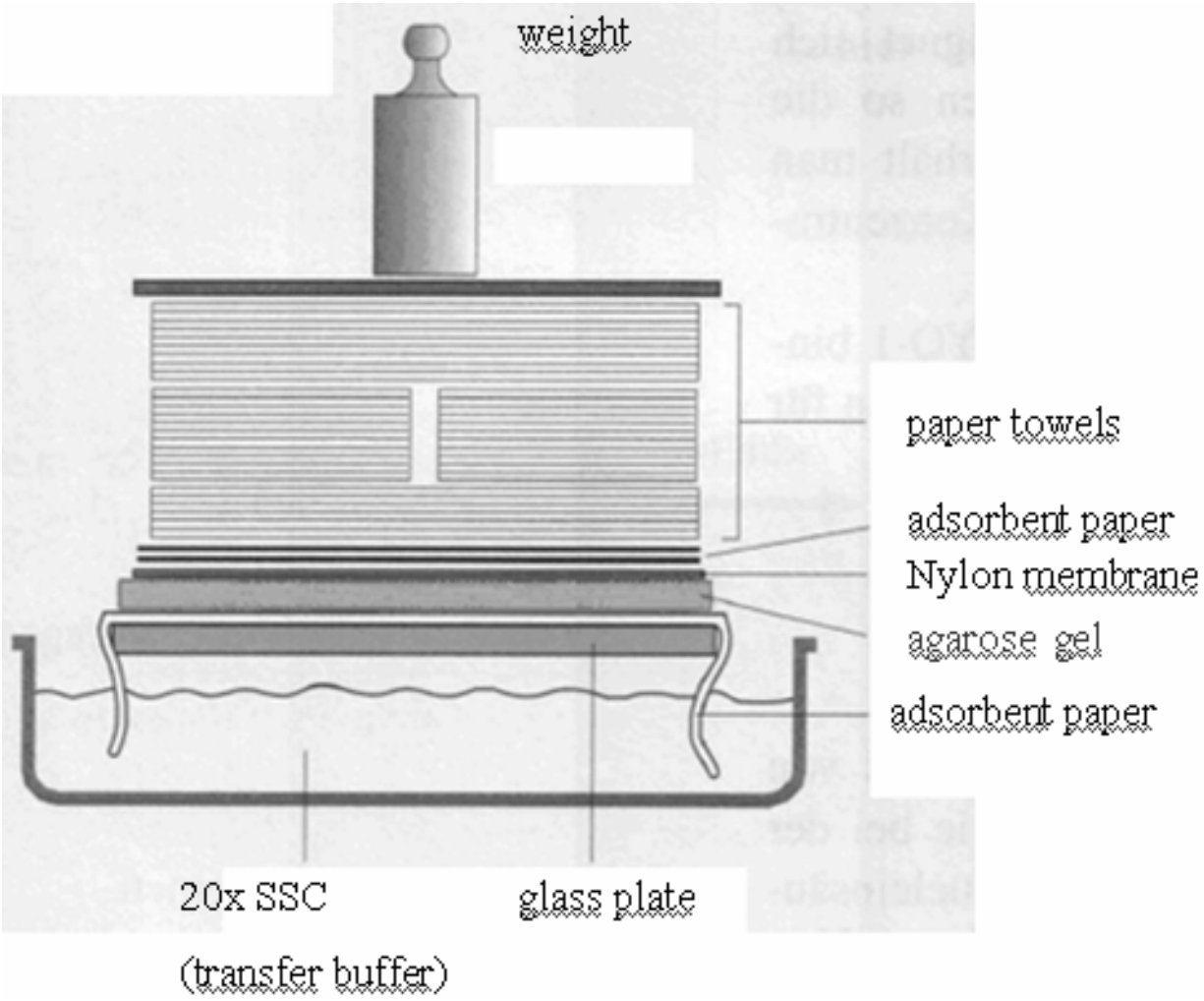


Figure 4.5 **capillary blot.** Transfer of DNA from agarose gel to a nylon membrane (Southern blot). Fig. was taken from: *http://www.uni-greifswald.de/~genetik/GenetikII/5Analytik.pdf* and modified.

4.8.2.4 UV-Crosslink

After blotting, the semi-dry membrane is placed into the UV-crosslinker (Stratalinker UV Crosslinker, Stratagene) and the DNA was cross-linked two times at 1,200J. The cross-linked membrane is washed in 2xSSC.

4.8.2.5 Hybridisation, chemoluminescence-detection and meanTRF calculation

The membranes are placed in a glass tube of the hybridzation ofen (Shake `n´ Stack, Hybaid). Membranes are placed between meshes and loaded in the tube as described be the manufacturer and pre-hybridised for 30 minutes at 42°C in 18 ml of prehybridisation solution (TeloTAGGG Telomere Length assay, Roche). The membranes were then hybridised for three hours in 6.5 ml of freshly prepared hybridisation solution at 42°C.
After hybridisation, the membrane is washed 2 times with sufficient stringent washing buffer I for 5 minutes at room temperature, and two times with sufficient prewarmed stringent washing buffer II for 15 minutes at 50°C. Membrane is transferred from tube to a tray and washed in 100 ml of washing buffer for 5 minutes. After washing the membrane in 100ml washing buffer, it is incubated in 100 ml of freshly prepared blocking solution. While the membrane is blocked, the antibody working solution is prepared.
The blocking solution is then displaced by the antibody working solution and the membrane is incubated for 30 minutes at room temperature. Again wash the membrane two times for 15 minutes in 200ml of washing buffer and incubate the membrane for 2-5 minutes at room temperature in detection buffer.
Place the membrane on adsorbent paper and immediately place it on an opened transparent envelope bag. Put 40 drops (3ml) of substrate solution (CDP-Star) on the membrane and close the hybridisation bag, and incubate for 5 minutes at room temperature. Discard the excess of substrate solution and place it for 20 minutes on an X-ray film or on two staged films to get different grey scale saturations at once.

After that the blot is visualised on Chemi-Smart 5100 analysing system (BioRad) and processed with ChemiCapt software. Mean TRF were determined in kbp with ImageQuant 5.0 software (Molecular Dynamics) according to the manufacturer's instructions of the *T*elo*TAGGG* Telomere Length Kit (Roche). The exposure picture of the blot was derived from ChemiSmart visualisation system as TIFF file and was loaded first in ImageJ 1.42 software (NIH) for background correction (with rolling ball radius 200 pixel) and saved as 16bit TIFF file.

This TIFF file was the reopened in ImageQuant 5.0 and a grid with 30 boxes is laid over every lane (including lane for the visualised molecular weight marker). The signal intensity is documented by volume report for the boxes. For each band of the molecular weight marker the length of migration is documented (for example the band corresponding the 21kb molecular weigth was found between box 8 and 9 at a box value of 8.2). By interpolation the molecular weight is calculated for every box of the lanes.
And with the formula

$$\text{mean}TRF = \sum (OD_i) / \sum (OD_i/L_i)$$

the mean TRF length is calculated. In this formula, ODi is the chemiluminescent signal and Li is the length of the TRF fragments at position i. This formula takes in account the higher signal intensities from larger TRF fragments because of multiple hybridisations of the telomere-specific probe [38].

4.9 Subtelomeric DNA methylation

4.9.1 Bisulfite DNA conversion

DNA conversion was performed with EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. DNA isolated by TRIZOL and Maxwell method was used. Thaw DNA samples for Bisulfite DNA conversion. Dissolve Bisulfite Mix by adding 800 µl RNase-free water and vortex until Bisulfite Mix is completely dissolved. The reactions are prepared in 250µl PCR tubes as described in Table 4.7.

Table 4.7 **Bisulfite conversion of DNA**

Component	Volume per reaction (µl)
DNA	1µg
Rnase-free water	variable
Bisulfite Mix	85
DNA Protect Buffer	35
Total volume	140

The combined volume of DNA and nuclease-free water must total 20µl, DNA concentration should be at least 50ng/µl

The reaction is mixed and the colour of DNA protect buffer should turn from green to blue after addition, indicating the correct pH in the reaction mix. The bisulftite conversion is performed on a thermal cycler HYBAID Omn-E (GenXpress), using the following thermal profile (Table 4.8).

Table 4.8 **Thermal profile for bisulfite conversion of DNA**

Step	Time minutes	Temperature °C
Denaturation	5	99
Incubation	25	60
Denaturation	5	99
Incubation	85	60
Denaturation	5	99
Incubation	175	60
Hold	indefinite	28

DNA conversion was performed on HYBAID Omn-E thermal cycler (GenXpress)

After conversion is complete, the converted DNA is cleaned up. Therefore, the reaction tubes are briefly centrifuged, and the reaction mix is transferred to a 1.5 ml tube (Eppendorf). 560µl of loading buffer (Buffer BL) are added and mixed by vortexing. The whole mixture is transferred to an EpiTect spin column and all following steps are processed at RT.
The column is centrifuged at maximal speed for 1 minute. The flow-through is discarded and 500 µl of washing buffer (Buffer BW) are placed on the spin column. Column is again centrifuged at maximal speed for one minute, and the flow-through is discarded. 500 µl desulfonation buffer (Buffer BD) are loaded on the spin column and incubated for 10 minutes. Column is again centrifuged for 1 minute at maximum speed, and flow through is abolished. Then, the spin column is washed two times with washing buffer.
Collection tube is replaced by a fresh one and spin column is centrifuged for 1 minute at maximum speed to remove residual liquids. DNA is eluted from the spin column with 20µl of elution buffer (Buffer EB) by placing the column on a 1.5ml tube. Tube is centrifuged for 1 minute at 15,000 g. To increase the yield of DNA, a second time 20 µl of elution buffer is applied to the column and collected by centrifugation. At the end 40 µl of DNA is obtained, mixed, spinned down and stored at -20°C until use.

4.9.2 PCR amplification of bisulfite converted DNA

PCR was performed on iCycler (Bio-Rad) with a touchdown PCR protocol as described [24]. For methylation-specific PCR of the chromosome 2p-subtelomer and 18p-subtelomere region 20 ng bisulfite converted DNA was used in a total of 20 µl reaction mixture containing 100 nM primer HC_MP2 FWD and 100 nM of a modified primer HC_MP2 REV_S (Table 4.4 and Table 4.5).

Table 4.9 **PCR conditions for amplification of bisulfite converted DNA**

PCR phase	Temperature °C	Time	Number of cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	10
	62 ->52*	45 seconds	
Annealing/extendsion	94	30 seconds	28
	62	45 seconds	
Melting curve	72	5 minutes	1

*touch-down PCR protocol: annealing temperature reduced by 1°C at each cycle

PCR fragments of 156 bp in size were purified by 3% agarose (Biozyme) gel electrophoresis. The extent of methylation was determined by ordered direct sequencing of PCR fragments by QIAGEN GmbH, Hilden, as follows:

All sequencing reaction mixes were based on the BigDye™ 3.1 Terminator chemistry (Applied Biosystems). Template amounts of 5 ng per 100 bases of fragment-length and 10 pmol primer per reaction were used. Reactions were cycled in a GeneAmp® PCR System 9700 (Applied Biosystems) and purified using DyeEx™ (QIAGEN).

Data collection was carried out on a 3730xl DNA Analyzer (Applied Biosystems) equipped with 50 cm capillary arrays and POP 7™ polymer. After data collection the raw data channels from the generated result-files (.ab1) were processed using custom-built software developed at QIAGEN. Data processing involved the compensation of the different migration-properties of the four dyes, baseline correction, peak detection and base-calling. For each base the area was calculated from the corresponding peak to measure the base's quantity.

Bases that correspond to variable positions of CpG-sites were identified by comparison of the obtained sequence with a reference sequence. The ratios of methylated versus unmethylated species were reported. Validation experiments have shown that the accuracy is in the range of ±5 % when comparing differences obtained for the same CpG-site from different samples having a signal-to-noise ratio of 20 or above.

5 Results

28 GBM, 6 AA and 12 DA tissue samples from patients with known diagnosis were analysed for telomere length. Furthermore, 6 non-tumour tissue samples from 2 patients and a subset of 12 human tumour cell lines originated from astrocytomas plus one from each meduloblastoma and osteosarcoma were analysed.

5.1 Results of DNA isolation

DNA was isolated by two different methods, from Trizol and form Chaps remainders as described in Materials and Methods chapter 4.2.
The two methods for isolating DNA were compared by quantifying the resulting DNA. This was done, using two different methods for quantifying DNA, NanoDrop ND-1000 Spectrophotometer (Peqlab) and Qubit Fluorometer (Invitrogen, Fisher Scientific). DNA was isolated from parts of the same frozen tissue with comparable size. Tissue was cut semi-frozen in pieces as described in Methods 4.2.1 Homogenisation of tissues.

The results from 4 analysed tissues showed, that the amount of DNA isolated from Chaps remainder was up to the 1-3 fold, compared to DNA isolated from Trizol (Table 5.1).
When the DNA isolated from Trizol was quantified, the results showed with Qubit fluorometer a strong decreased amount as compared to the Nanodrop measurement. The Quibit method recognizes exclusively intact dsDNA, while the photometer does not. Because of that, the amount of DNA isolated from Trizol was strong overestimated, potentially because of free nucleotides, which are measured by Nanodrop, but not with Qubit fluorometer. However, for DNA, isolated from Chaps remainders, the amount of DNA was similar or slightly higher, when quantified with Qubit fluorometer. Thus reflects a higher quality of intact dsDNA isolated from Chaps remainders as compared from Trizol.

Furthermore, the amount of contaminating proteins and RNA was also determined with Qubit fluorometer in DNA samples. Proteins could not be detected in any of the DNA samples (detection limit of protein <12.5µg/ml). 260/280 ratio indicates higher amount of contaminating proteins in DNA samples isolated from Trizol (see Table 5.1).
In contrast to proteins, RNA could be determined in all DNA samples. The ratio of RNA to DNA was calculated to be about 100-times higher, when the DNA was isolated from Chaps remainders with Maxwell DNA tissue DNA purification kit, when compared to Trizol

isolated DNA. This seems to be due to the lower amount on DNA in Trizol lysats, because the amount on RNA is in both methods of isolation very similar.
When DNA was isolated from the Chaps remainder and from a piece of frozen tissue with Maxwell tissuec DNA purification kit, the amount of isolated DNA was also higher in Chaps remainders (up to the 2 fold).

Table 5.1 **Comparison of different methods of DNA isolation (Trizol or Chaps) and quantification (Nanodrop or Qubit)**

	nanodrop			**Qubit**			
Sample		**total**			**total**	**RNA total**	**RNA/DNA**
	ng/µl	**µg**	**260/280**	**ng/µl**	**µg**	**µg**	
GBM21_T	183.14	**9157**	1.56	6.7	**335**	1.315	1:255
GBM21_T	34.07	**10221**	1.74	47.2	**14160**	1.25	1:11328
GBM20_T	360.14	**18007**	1.83	2.3	**115**	2.805	1:41
GBM20_C	105.86	**31758**	1.86	120.2	**36060**	3.475	1:10377
GBM28_T	33.34	**1667**	1.64	2.3	**116**	to low	
GBM28_C	74.85	**22455**	1.9	98.2	**29460**	2.685	1:10972
GBM19_T	66.08	**3304**	1.75	3.1	**156**	1.765	1:88
GBM19_C	31.36	**9408**	1.88	39.2	**11760**	1.41	1:8340

For Nanodrop and Qubit fluorometer the concentration is given in ng/µl and the total amount of DNA is calculated. Trizol DNA was dissolved in 50µl of NaOH buffer and Qubit DNA was dissolved in 300µl of elution buffer (EB).
T … Trizol;
C … Chaps;

Tumour cells require a telomere maintenance mechanism, usually provided by the enzyme telomerase, but some of telomerase negative tumour cells can maintain their telomeres by a mechanism known as alternative lengthening of telomeres (ALT), which is based on recombination events. It was one goal of this study, to measure the telomere length of astrocytoma tumour samples and astrocytoma cell lines (telomerase positive examples) and the osteosarcoma cell line Saos-2 (as an example for an ALT cell line). Saos-2 is a telomerase negative cell line and utilizes ALT mechanism. One hallmark of this mechanism is the extremely long and heterogeneous telomere length in contrast to cells that use telomerase activity. The telomere lengths were measured by terminal restriction fragment (TRF) analysis and by a quantitative real-time PCR assay as described in Materials and Methods.

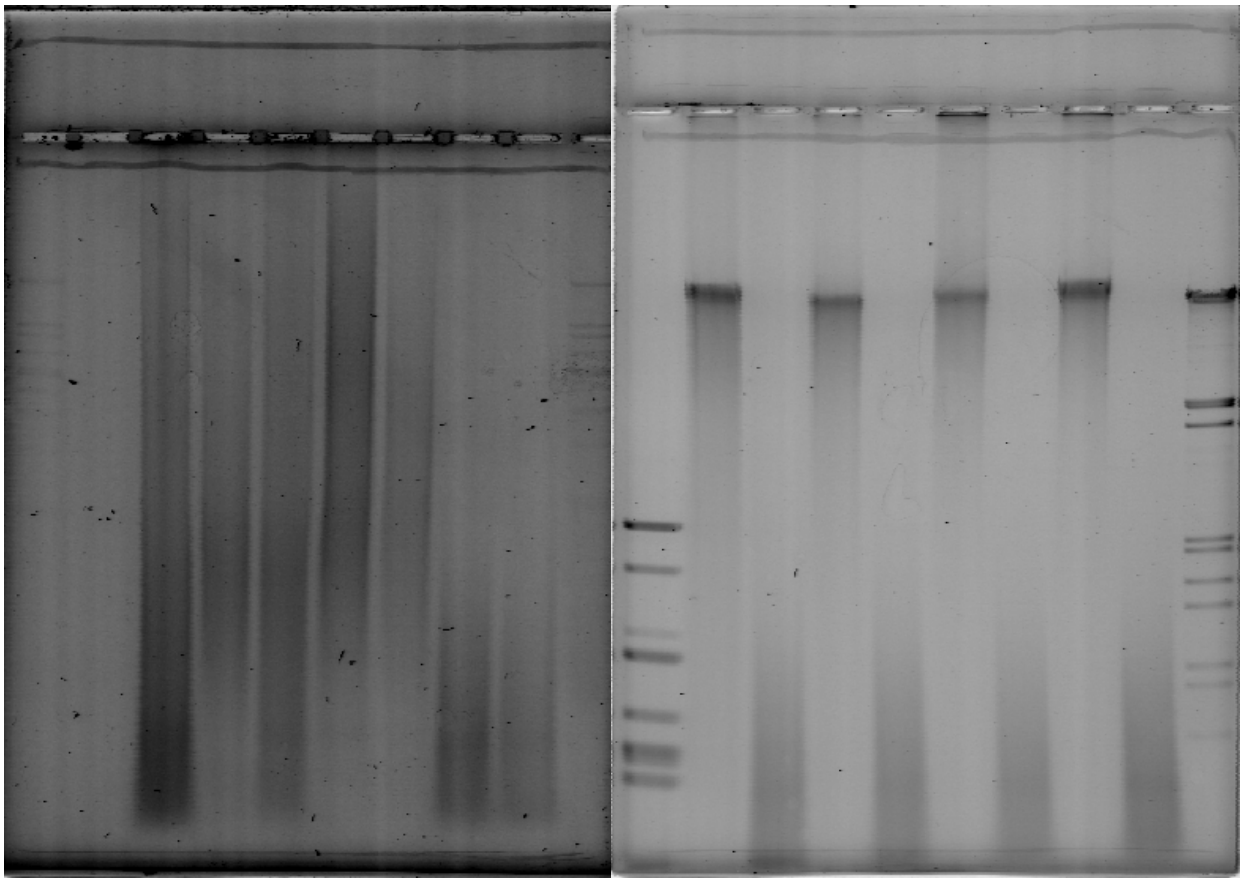
5.2 Telomere lengths ananlysis

Telomere length was determined by a quantitative real-time PCR assay with primers tel1b und tel2b for amplification of telomeric TTAGGG–repeats (T), and with primers 36B4d and 36B4u for comparision with a single copy gene 36B4 (S). PCR conditions are described in Table 4.4 of chapter Materials and Methods. Relative telomere length was determined as RQ value (T/S). First, telomere lengths of a subset of 14 tissue samples and 5 cell lines were analysed and compared with the two methods, terminal restriction fragment (TRF) analysis and quantitative real-time PCR.

5.2.1 Terminal restriction fragment analysis

Terminal fragment length analysis was performed with *T*elo*TAGGG* Telomere Length Assay kit (Roche) as described in detail in Methods. Genomic DNA isolated from cell lines and tumour tissue samples was digested with restriction enzymes *Rsa*I and *Hin*fI, size fractioned on agarose gel, stained with ethidium bromide and visualized on the FluorImager 595 (Figure 5.1). As we tested, DNA isolated from Trizol was only partially digested, even when digested overnight in a larger volume (instead of 20 µl 100 µl total volume set up was applied). So we used Chaps remainders as described in literature [30] and isolated DNA with the automated Maxwell 16 tissue DNA purification system. This DNA could be completely digested, and was used for all TRF analysis done within this study.

a) b)



1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10

Figure 5.1(a + b) **Digestion of genomic DNA with restriction enzymes *Rsa*I and *Hin*fI.** Samples with 1 µg DNA were analysed by 1% agarose gel electrophoresis, stained and visualized on FluorImager.
(a) DNAs isolated from Trizol are digested only partially. Only the two control DNAs from the TeloTAGGG Telomere length kit are completely digested (lane 8 + 9), GBM samples isolated from Trizol are (Bitte immer die selbe Zeit nehmen!) only partially digested (lanes 2 - 7). Marker from Roche TeloTAGGG kit is loaded in lane 1 and 10.
(b) DNAs isolated with Maxwell 18 tissue DNA purification kit are completely digested. Undigested (lanes 2, 4, 6, 8) and digested DNA (3, 5, 7, 9) from the same tissue probe are loaded on the gel in adjacent lanes. Lanes 1 and 10: markers

Two control DNAs of the Telo TAAGGG kit (control-DNA low and control-DNA high) with known meanTRF lengths and a Digoxigenin- (DIG-)labeled DNA molecular weight marker were also loaded on the agarose gel (see Figure 5.2.a). For the two control-DNAs, included in the kit, the manufacturer gave a mean TRF length of 3.9 kb for control low DNA and 10.2 kb for control high DNA.

After electrophoresis, gels were blotted, hybridised with a DIG-labelled telomere-repeat specific probe and detected with an anti-DIG antibody, linked to alkaline phosphatase. This phosphatase cleaves the applied substrate CDP star, leading to the production of light.

The results where visualized on Chemi-Smart 5100 analysing system (BioRad) and further on X-ray films.

On first experimental blots, only cell culture cells were analysed (Figure 5.2).

To test the sensitivity for the detection of TRF targets from the assay, a 1:5 dilution series of the included control high mean TRF DNA were applied in amounts of 760 ng, 200 ng and 40 ng (figure 5.2). TRF signals with low amount of 40 ng DNA could be still detected, but the signal intensity was very weak.

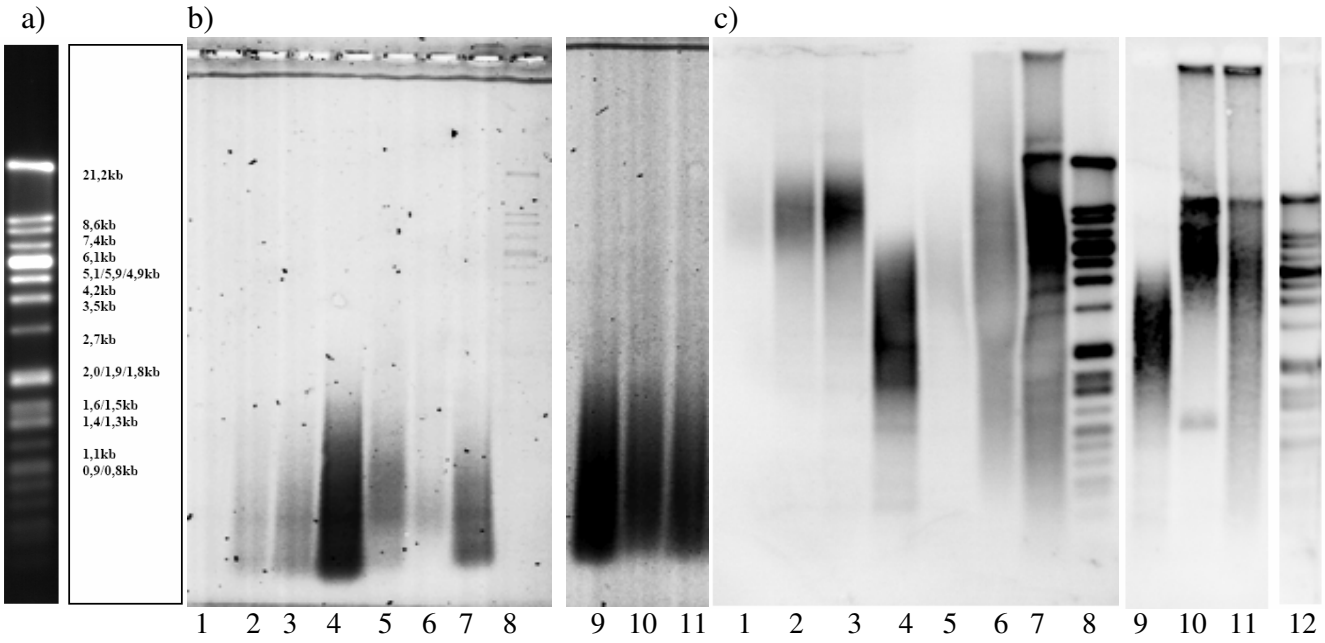


Figure 5.2 (a-c) **Analysis of terminal restriction fragment (TRF) length for cell lines.**

(a) λ-DNA with EcoRI digested and indicated fragment length (included in the kit)

(b) genomic DNA (isolated with Maxwell 16 tissue DNA purification kit) was digested with *Rsa*I und *Hin*fI and size fractioned on a 1% agarose gel, stained with ethidium bromide and visualized on FluorImager 595. 40 ng of control high (lane 1), 200 ng of control high (lane 2), 760 ng of control high (lane 3); control low (lane 4), HeLa (lane 5), Saos-2 DNA isolated from Trizol (lane 6), Saos-2 DNA isolated with Maxwell system (lane 7), λ-Marker (lane 8), MR1 (lane 9), YT-BO (lane 10), KM-HY (lane 11)

(c) Southern blot of digested genomic DNA. 40 ng of control high (lane 1), 200 ng of control high (lane 2), 760 ng of control high (lane 3); control low (lane 4), HeLa (lane 5), Saos-2 DNA isolated from Trizol (lane 6), Saos-2 DNA isolated with Maxwell system (lane 7), λ-Marker (lane 8), MR1 (lane 9), YT-BO (lane 10), KM-HY (lane 11) and λ-Marker (lane 12)

In next experimental blots, DNA purified from tumour tissue samples were analysed (Figure 5.3). One sample originates from a tumour grade II (DA), one sample from a tumour grade III (AA) and 14 samples are from tumours diagnosed with grade IV (GBM).

As an additional control to test for proper digestion, one of the GBM probes was additionally applied without being digested before. The results of this control is shown on the gel and blot by an almost defined single band at 21 kb (Figure 5.3 a+b, GBM 11 on lane 15). The same

DNA probe was digested to a low molecular weight DNA smear (Figure 5.3a: GBM 11 on lane 16) and results on the blot lower than 21 kb TRF (Figure 5.3b: GBM 11 on lane 16). This control demonstrates the importance for testing of proper digestion to measure correct TRF signals.

Both, the grade II and the grade III sample show long telomeres on the blot, but there are also GBM samples with long telomeres (see Figure 5.3 b, lanes 3, 5 and 16 GBM with long telomeres).

a) b)

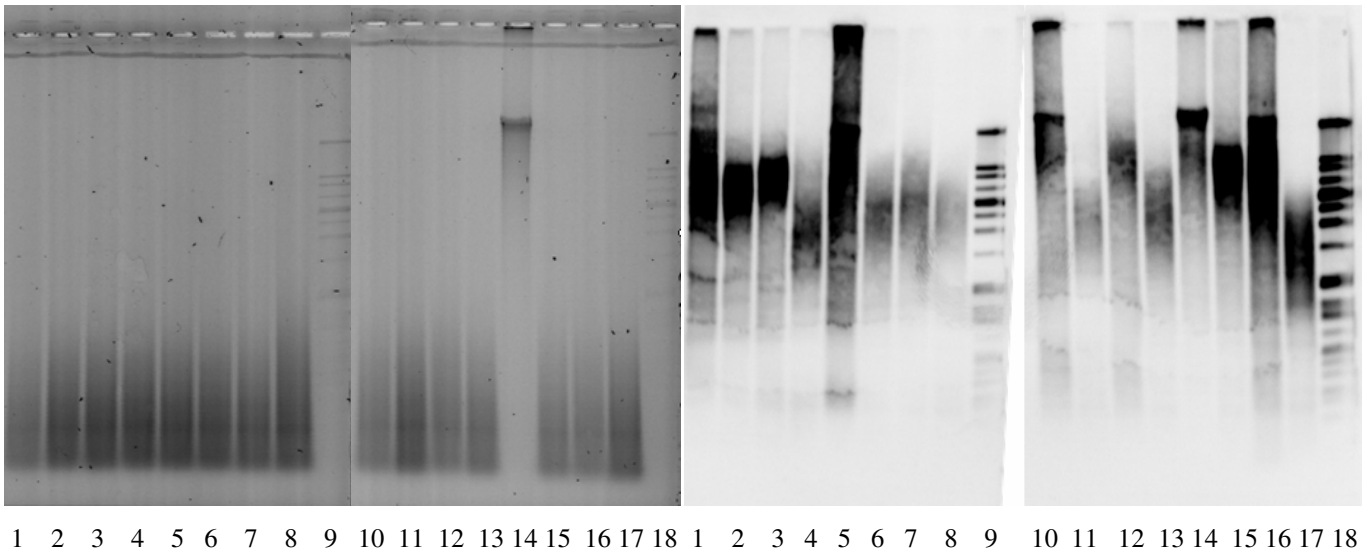


Figure 5.3(a+b) **Analysis of the terminal restriction fragment (TRF) length for tissue samples.**
(a) Genomic DNA was digested with *Rsa*I and *Hin*fI (except lane 14 is undigested), size fractioned on a 1% agarose gel, stained with ethidium bromide and visualized on FluorImager. AA 1 (lane 1), GBM 4 (lane 2), GBM 23 (lane 3), GBM 2 (lane 4), GBM 27 (lane 5) GBM 25 (lane 6), GBM 20 (lane7), GBM 28 (lane 8), λ-Marker 2µg (lane 9), DA 10 (lane 10), GBM 16 (lane 11), GBM 2 (lane 12), GBM 14 (lane 13), GBM 11 undigested (lane 14), GBM 11 (lane 15), GBM 10 (lane 16), control low (lane 17), λ-Marker 4µg (lane 18)
(b) TRF Southern blot result of DNA from tissue samples. AA 1 (lane 1), GBM 4 (lane 2), GBM 23 (lane 3), GBM 2 (lane 4), GBM 27 (lane 5) GBM 25 (lane 6), GBM 20 (lane7), GBM 28 (lane 8), λ-Marker 2µg (lane 9), DA 10 (lane 10), GBM 16 (lane 11), GBM 2 (lane 12), GBM 14 (lane 13), GBM 11 undigested (lane 14), GBM 11 (lane 15), GBM 10 (lane 16), control low (lane 17), λ-Marker 4µg (lane 18)

Hybridisation of TRF-blots with a single copy gene probe for normalisation:

A DIG-probe for a single copy gene was applied to test the hypothesis if this can detect *Rsa*I and *Hin*fI restriction fragments for proper normalisation of DNA amounts applied.

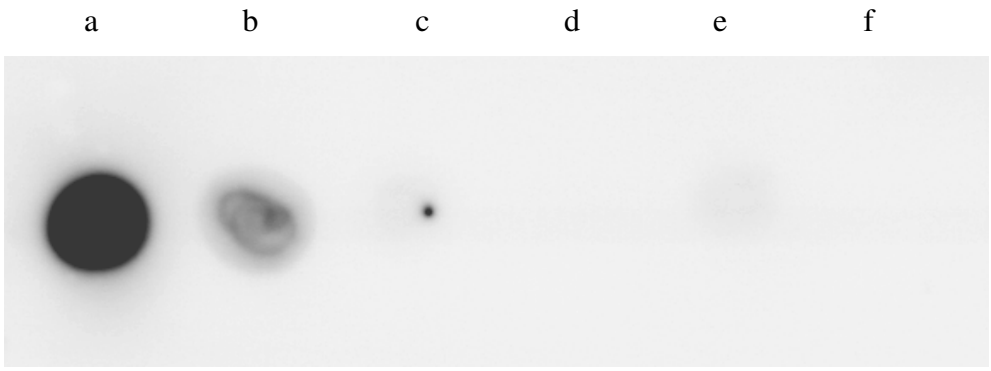


Figure 5.4 **Dot blot of DIG-labeled probe for gene 36B4**

Probe was prepared as described under methods and diluted 1:10 (a), 1.100 (b), 1:1,000 (c), 1:10,000 (d) in 2xSSC, PCR mix before cycling (e) and 2xSSC as a negative control (f) were spotted on the membrane.

A DIG-labelled probe for the housekeeping gene 36B4 was prepared. Dot blot was done to determine the efficiency of the DIG-labelling (Figure 5.4). Signals were detected at the 1:10 and 1:100 dilutions.

After the dot blot, the Southern blot (shown in Figure 5.3b) was rehybridized with this probe, but no signals were detected. This could be, because the amplicon for DIG-labeling is only 75 bp long and includes 21 T nucleotides, and only every 20th thymidine nucleotide is stochastically DIG-labelled. That means that statistically only around one thymidine nucleotide per amplicon is carrying a DIG-label.

This experiment can potentially be successfully repeated with more adequate labelled amplicons from single copy genes (e.g. beta actin, 800 bp in size).

Mean TRF analyses of tumour tissue samples and cell lines are summarized inTable 5.2.

Table 5.2 **MeanTRF length of tumour tissue samples and cell lines**

Sample	meanTRF (kb)	Sample	meanTRF (kb)
SAOS-2(C)	8.9	GBM 16	3.5
SAOS-2(T)	5.7	GBM 20	5.6
HeLa	3	GBM 23	12.3
MR1	2.5	GBM 25	6.7
KM-YH	4.8	GBM 27	9.5
YT-BO	8.8	GBM 28	5.6
GBM 2	4.8	AA1	25.4
GBM 4	8.8	DA10	29.8
GBM 10	17.2		
GBM 11	7.6	Control low	3.7
GBM 14	6.7	Control high	10.6

Results of terminal restriction fragment length analysis in astrocytoma tumor tissue samples and cell lines, including three astrocytoma cell lines MR1, KM-HY and YT-BO, cervical cancer cell line HeLa and osteosarcoma cell line SAOS-2. Cell lines are described in Materials and Methods.

5.2.2 Real time PCR assay for measuring telomere length

In a primary set up, three different thermal profiles were tested for the optimal measurement of relative telomere lengths on two GBM tumour samples. The first profil (60°C/1minute) was described recently in literatur [32]. The two other profiles were described in [31] for telomere amplification and amplification of 36B4, respectively. The three thermal profiles use different annealing temperatures and were tested for both the T and S assay (Table 5.3). The thermal profile with 60°C/1min showed the highest distance between Ct of sample and Ct of non-template control (NTC) for both T and S assays, so this thermal profile was used later to determine the relative telomere length by PCR.

Table 5.3 **Test of three thermal profiles for (T) and (S) PCR assay.**

PCR assay	60°C/1min				58°C/1min				54°C/2min			
	Ct	SD	Ct(NTC)	ΔCt	Ct	SD	Ct(NTC)	ΔCt	Ct	SD	Ct(NTC)	ΔCt
(T) Telomere	15.3	0.25	29.7	14.4	13.4	0.11	29.8	16.4	13.3	0,14	25.4	12.1
(S) 36B4	22.0	0.14	33.7	11.7	22.4	0.06	33.3	10.9	22.2	0.20	33.1	11.0
(T) Telomere	15.5	0.05	29.7	14.2	16.2	0.17	29.8	13.6	15.3	0.27	25.4	10.1
(S) 36B4	22.4	0.37	33.7	11.3	23.0	0.09	33.3	10.3	22.7	0.14	33.1	10.4

*(1) and (7) are tumour samples of astrocytic brain tumours (GBM1 und GBM7).

Ct … cycle threshold for the sample

SD … standard deviation

CtNTC … cycle threshold of non-template control

5.2.2.1 Determination of Efficiencies

For telomere length analyses a real time PCR assay was established (primers and PCR conditions are described in chapter Materials and Methods).
For (T) telomeric repeats and for (S) single copy or house keeping gene 36B4 the efficiency (E) was determined by standard curve experiments. DNA of plasmid pSP73 containing an insert with 120 telomeric repeats (800bp) were diluted in a 1:1 dilution series for template of the T standard curve. Similarily, plasmid pCRII-TOPO with cloned 36B4 amplicon was used for the S standard curve.
Number of molecules for standard curve are calculated [31], and for this purpose, conversion of pg of DNA to mol is done with DNA/RNA/Protein/Chemical Molecular Weight Calculator (http://www.changbioscience.com/genetics/mw.html), results given in table 5.4.

Table 5.4 **Dilution series for standard curves**

	Plasmid DNA (pg)	pSP73_tel (molecules)	pCRII-TOPO_36B4 (molecules)
1st step	10	298564887399	352961501992
2nd step	5	149282443699	176480750996
3rd step	2.5	74641221850	88240375498
4th step	1.25	37320610925	44120187749
5th step	0.625	18660305462	22060093875

Steps in dilution series are 1:1 dilutions of plasmid DNA and nuclease-free water
Number of molecules for pSP73 with insert of telomeric repeats and
pCRII-TOPO vector with inserted 36B4 gene are calculate

(a) (b)

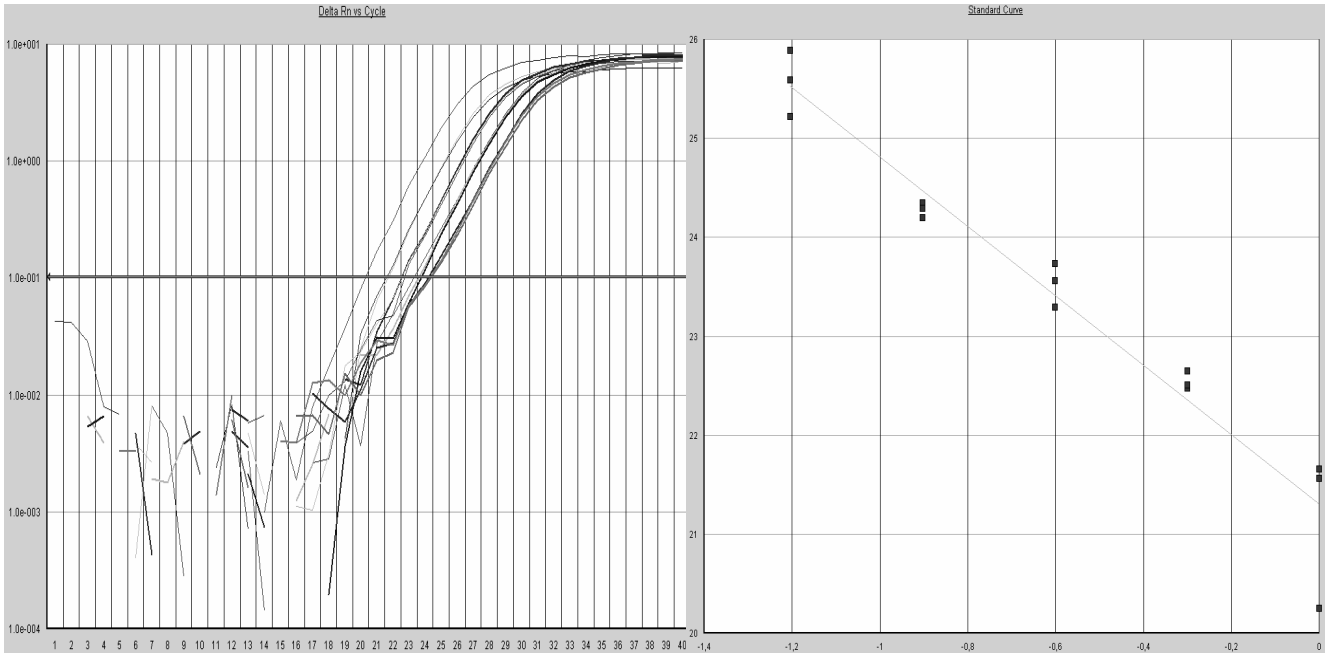


Figure 5.5 (a+b) **Standard curve for house keeping gene 36B4 measured on ABI 7000 PCR system**

(a) standard curve for 36B4 and

(b) amplification plot

(a) (b)

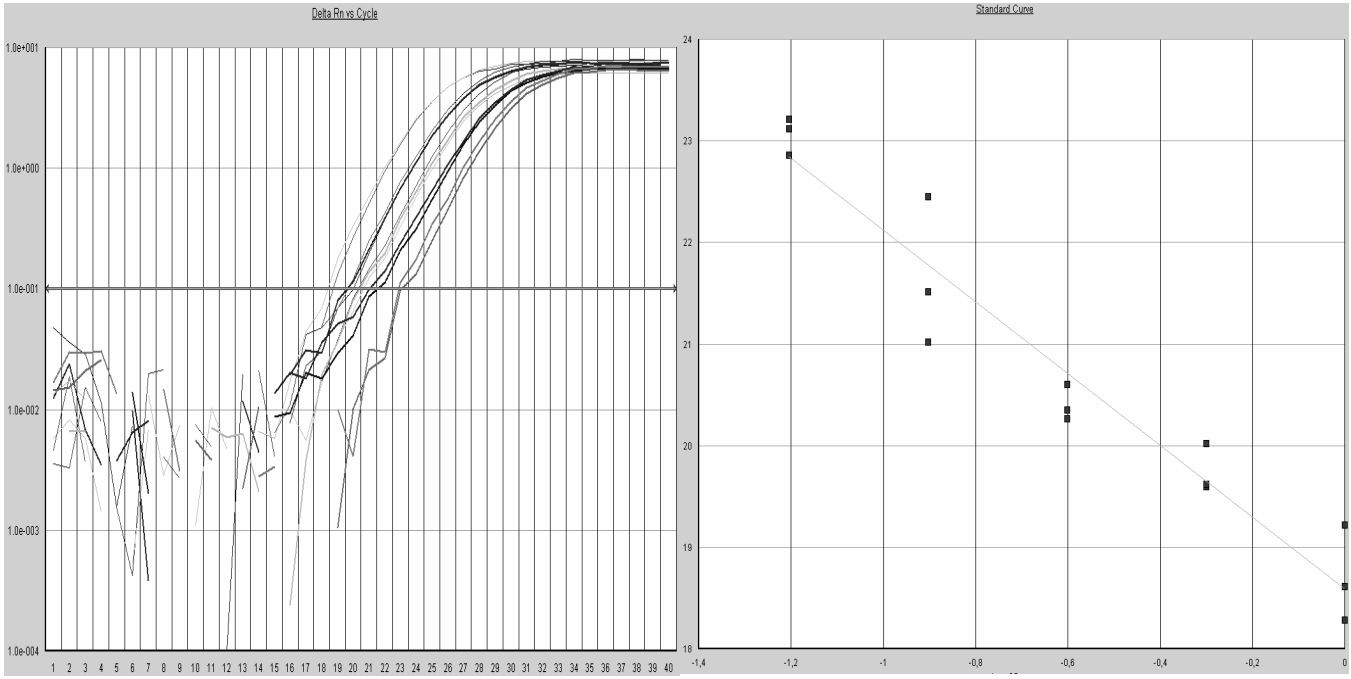


Figure 5.6 (a+b) **Standard curve for telomeric repeats on ABI 7000 PCR system**

(a) standard curve for telomeric repeats and

(b) amplification plot

(a) (b)

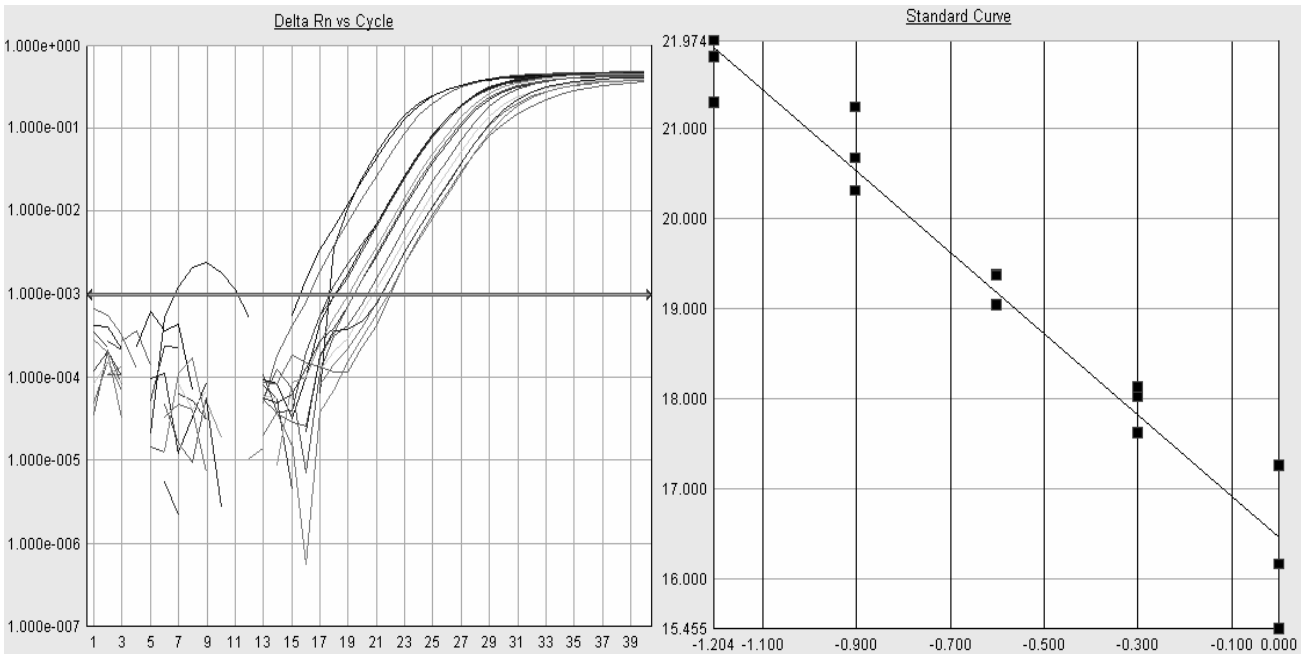


Figure 5.7 (a+b) **Standard curve for house keeping gene 36B4 on ABI 7500 Fast PCR system (with standard PCR program)**

(a) standard curve for 36B4 and

(b) amplification plot

(a) (b)

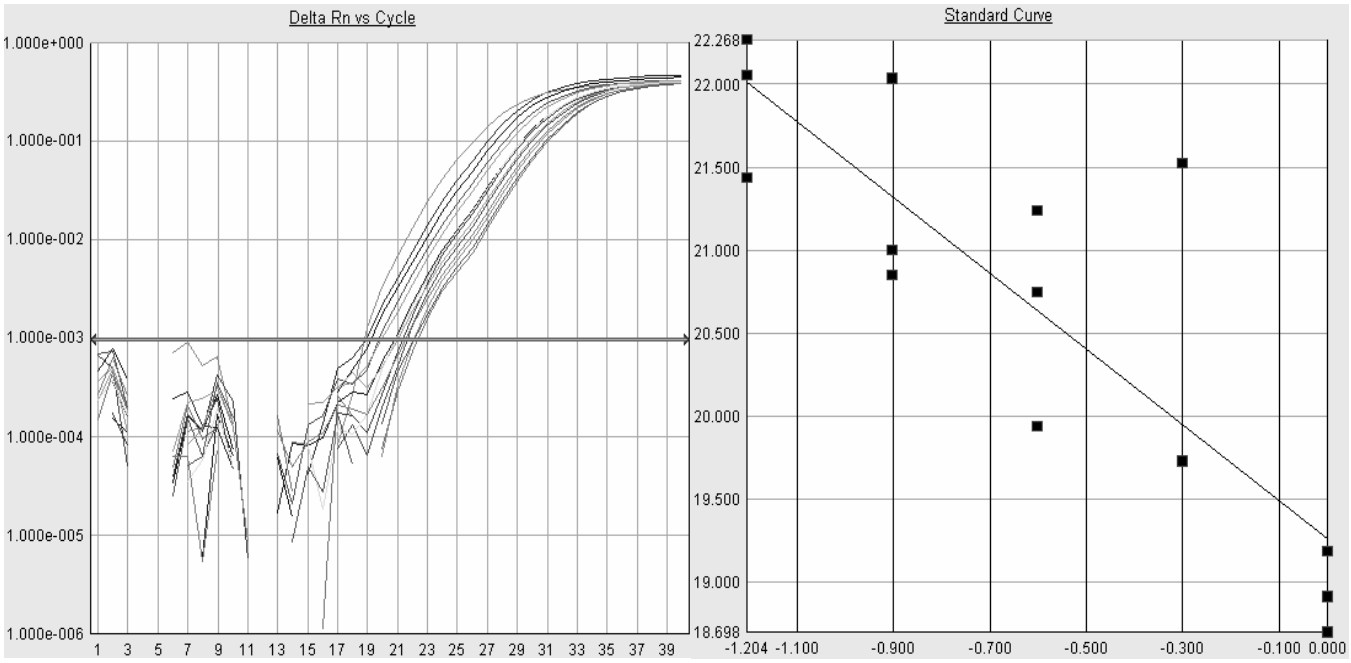


Figure 5.8 (a+b) **Standard curve for telomeric repeats on ABI 7500 Fast PCR system (with standard PCR program)**

(a) standard curve for telomeric repeats and

(b) amplification plot

(a) (b)

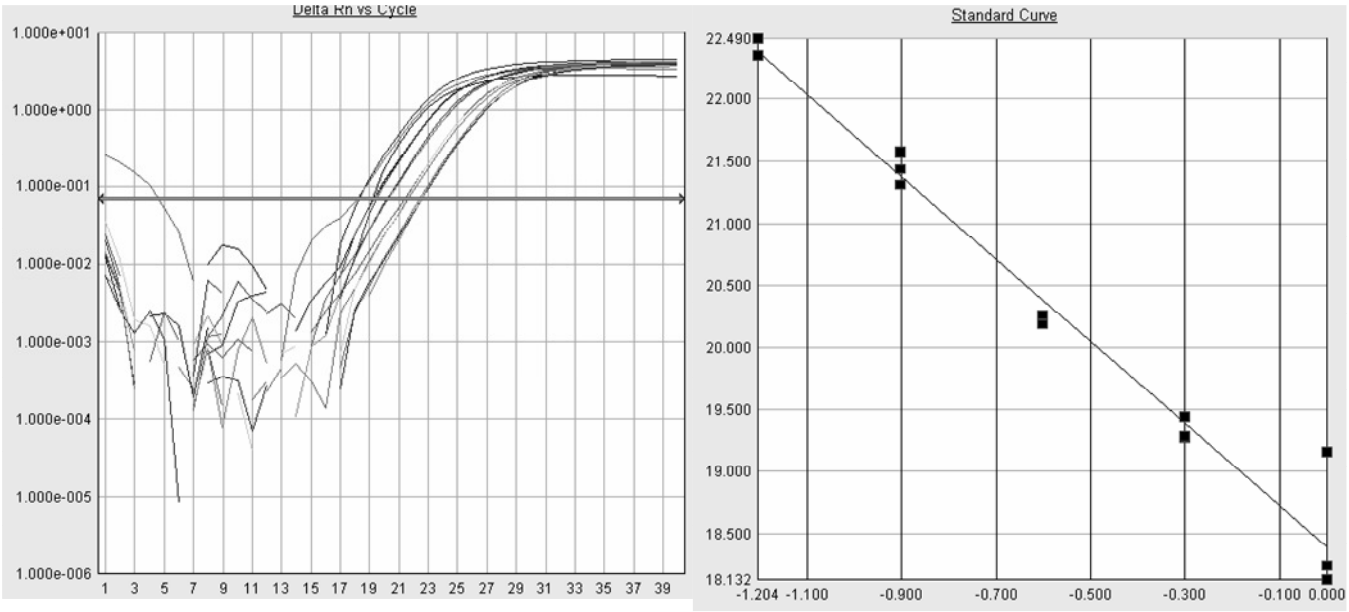


Figure 5.9 (a+b) **Standard curve for house keeping gene 36B4 on ABI 7500 Fast PCR system (with Fast program)**

(a) standard curve for 36B4 and

(b) amplification plot

(a) (b)

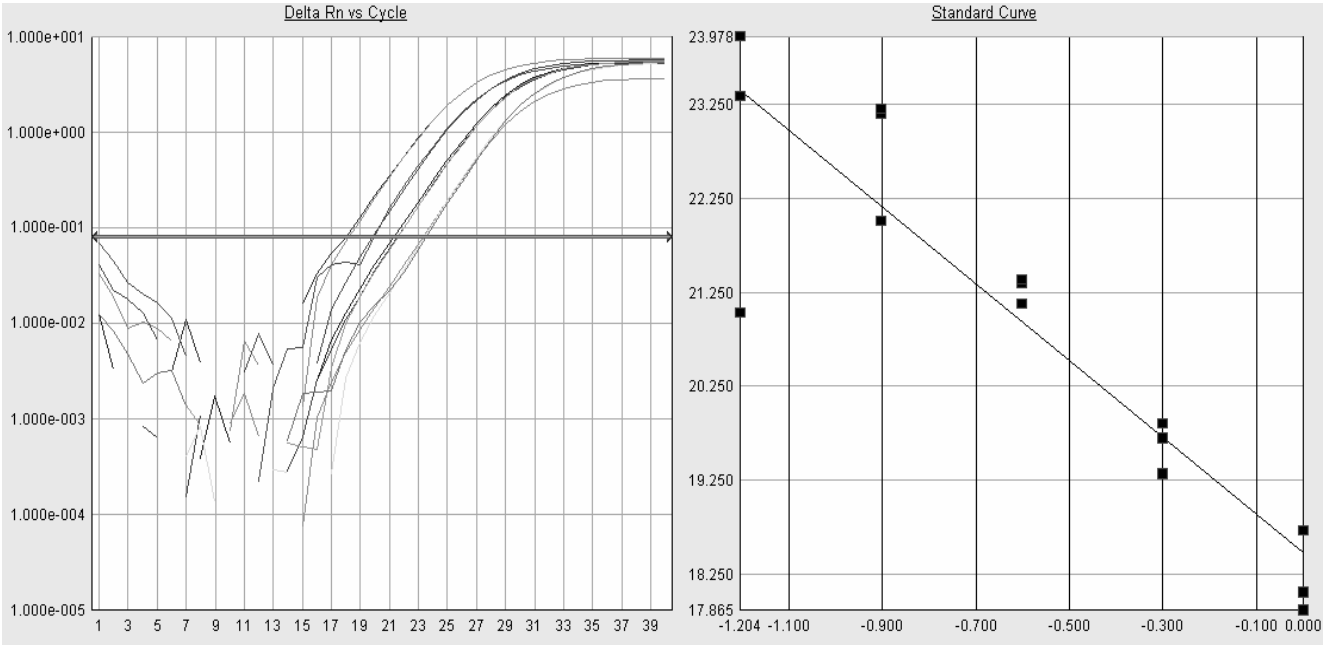


Figure 5.10 (a+b) **Standard curve for telomeric repeats on ABI 7500 Fast PCR system (with Fast program)**

(a) standard curve for 36B4 and

(b) amplification plot

Standard curves showed, that amplification of gene 36B4 is not very efficient, when amplified with standard program on 7500 Fast Real-Time PCR system (74%).

All other efficiencies were above 80% (for results see table 5.5).

Table 5.5 **Efficiencies for telomeric repeats and 36B4 tested on different systems**

		Slope	R^2	Efficiency (%)	Tm (°C)
7000	tel	3.535	0.946	94	79.3
	36B4	3.506	0.937	95	78.2
7500 st	tel	2.281	0.729	146	78.1
	36B4	4.513	0.949	74	78.1
7500 fast	tel	4.118	0.830	81	77.4
	36B4	3.314	0.971	100	76.1

Efficiencies on different systems and different chemistries (AB7000, AB7500 standard PCR, AB7500 fast PCR) were tested for single copy or house keeping gene 36B4 (S) and telomeric repeats (T).

5.2.2.2 Measurement of relative telomere length

After detection the efficiency of the assays, the relative telomere lengths were determined as relative quantity (RQ) values normalized to the single copy gene 36B4. Real time PCR assay for measuring telomere length was evaluated manually with a threshold cycle of 0.2 and baseline starts at Ct = 6 and ends at Ct = 15.

The baseline in the amplification plot is a line fit to the fluorescence levels during the initial stages of PCR, when there is little change in fluorescence signal. The threshold cycle (Ct) is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

Assay with primers tel1b and tel2b were tested on PAGE in Figure 5.11 and later run in triplicates. A smear with multiple tel products appears on gel. In contrast, the NTC developed a single product size (potentially a primer dimer) at late Ct values. If one of three Ct values showed a standard deviation (SD) > 0.5 it was excluded form the analysis. Assay with primers 36B4d and 36B4u were run in duplicates, and samples with SD > 0.5 were repeated.

a) b)

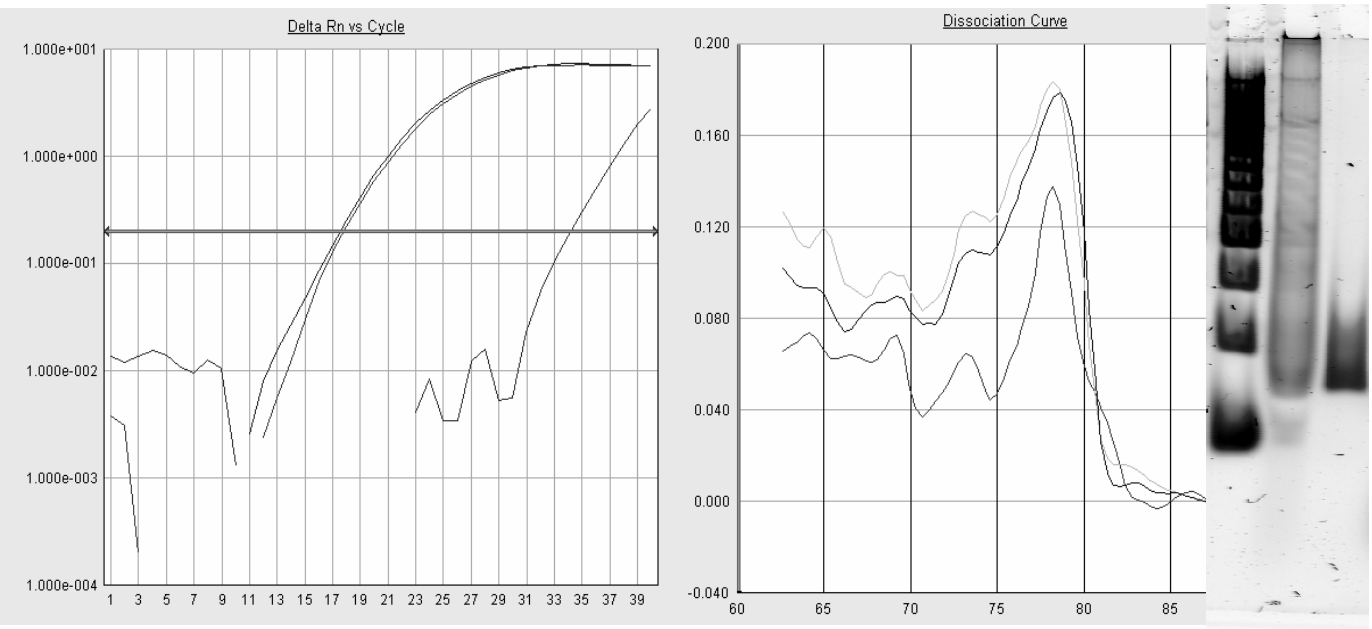


Figure 5.11 (a+b) **Amplicon of tel primers**

(a) show the amplification plot with number of cycles on the x-axis and Delta Rn on the y-axis

(b) show the melting curve of tel amplicons with the temperature on the x-axis and a PAGE gel with a 50 bp DNA ladder (lane 1), the tel amplicon (lane 2) and the non-template control (lane 3)

Primers for gene 36B4 were tested *in silico* with the CloneManager program by aligning them against the genomic sequence of the 36B4 gene. Sequence was taken from NCBI entry with the accession number NC_000012.11 (Figure 5.12).

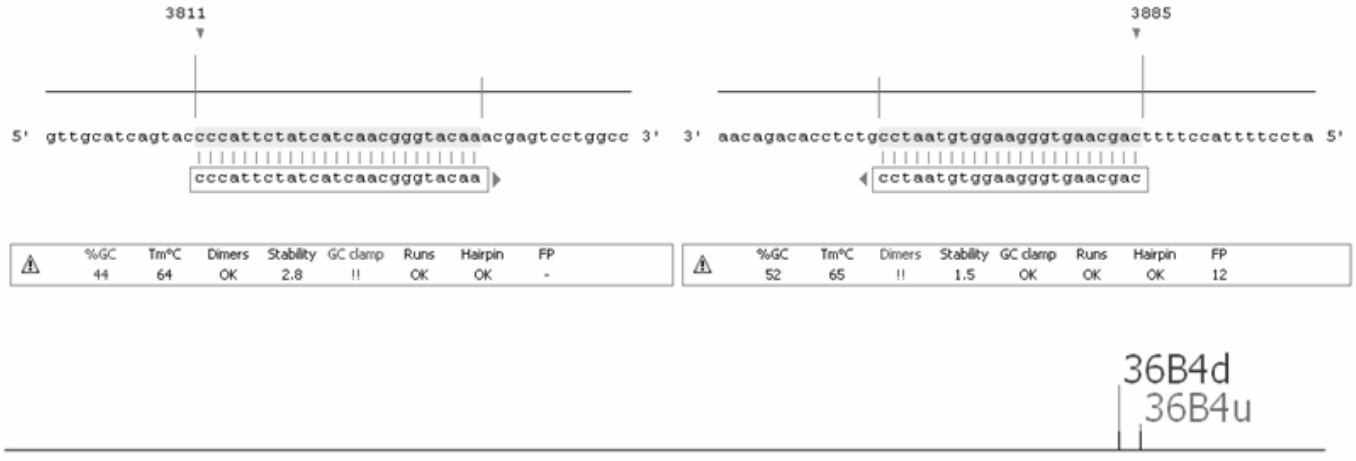


Figure 5.12 **Aligning of primers 36B4u and 36B4d to the genomic sequence of gene 36B4** in CloneManager 9 program (Primers are used for determining relative telomere length and expression of overall TERRA and 2p- and 18pTERRA)

For an additional test, the amplicon of gene 36B4 was analysed by PAGE. The amplicon shows the expected length of 75 bp and no product was detected in the non template control probe (Figure5.13).

(a) (b)

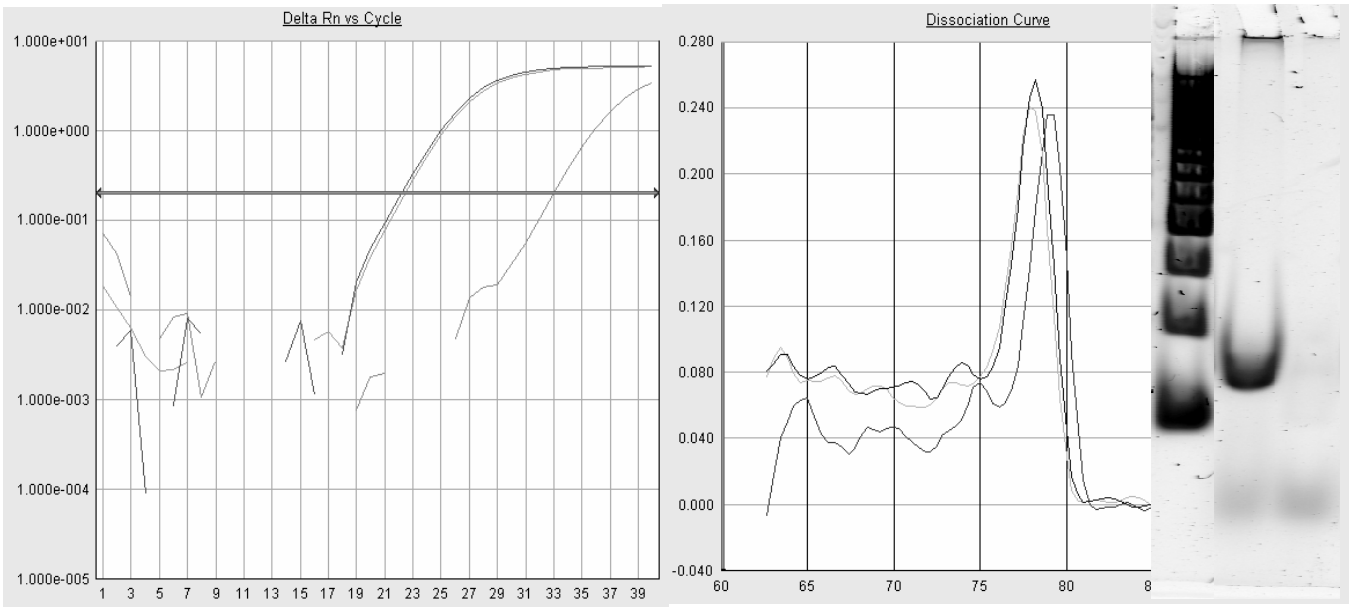


Figure 5.13 (a+b) **Amplicon of 36B4 primers**

(a) shows the amplification plot

(b) shows the melting curve of 36B4 amplicons and a PAGE gel with a 50 bp DNA ladder (lane 1), the 36B4 amplicon (lane 2) and the non-template control (lane 3).

For statistical analyses, RQ values were imported into GraphPad Prism 5. First, RQs were tested for normal distribution (D'Agostino and Person omnibus Test, Kolmogorow-Smirnov- und Shapiro-Whilk Test).
RQ values were than tested with the Mann-Whitney test for significant differences in the median of different groups (e.g. tumour grades).

RQs for telomere lengths are shown in Figure 5.14 for GBMs, DAs, AAs, non-tumor tissue and cell lines (10 derived from astrocytomas, 1 from a primary osteosarcoma and one from a medulloblastoma). RQ values were related to the median of non-tumour tissue. Tumour tissues and cell lines demonstrate 7.6 to 32.3-fold increase in relative telomere length. The shortest telomere lengths were observed in non-tumour tissues and sporadic in single astrocytomas (DA5, RQ=0.36 and GBM9, RQ=0.5) and cell lines (MGC, RQ=1.04). Cell lines showed high telomere length comparable to low grade II and III tumours.
RQ values of telomere length demonstrate to be significantly increased (p=0.0014 for AA and p=0.0033 vor DA) in tumours with grade II and grade III (DA + AA) when compared to high

grade tumours (GBM), but there was no significant difference between grade II and grade III (Figure 5.14).

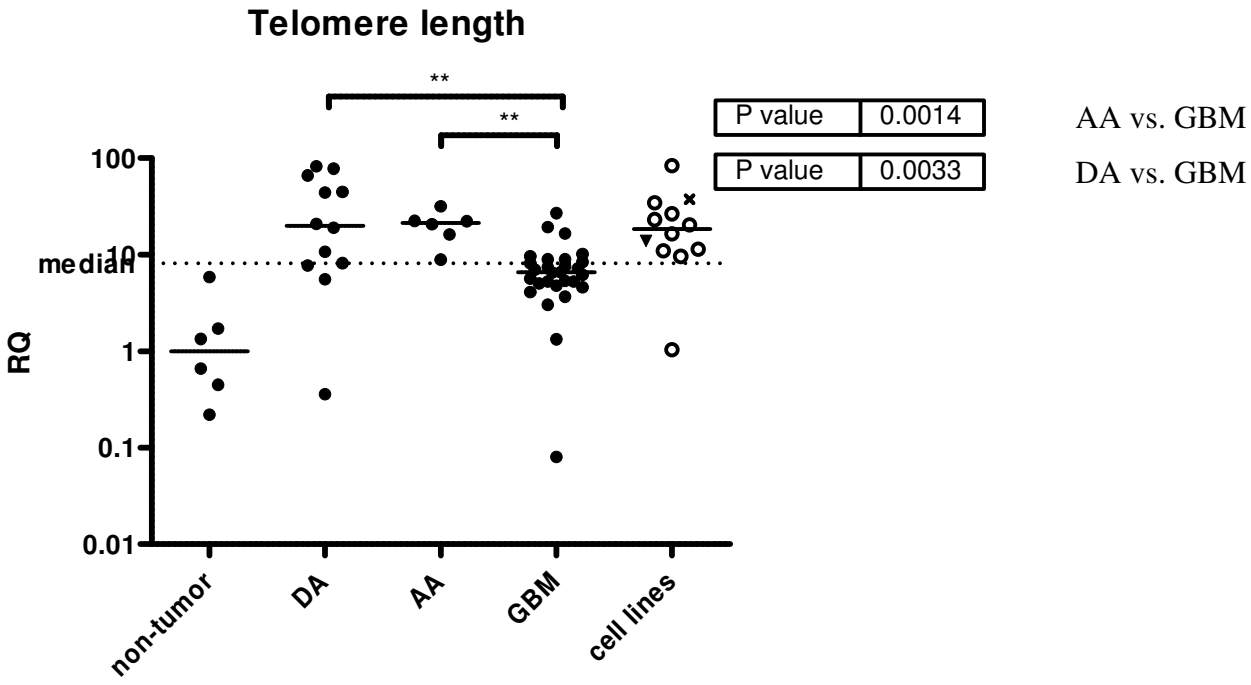
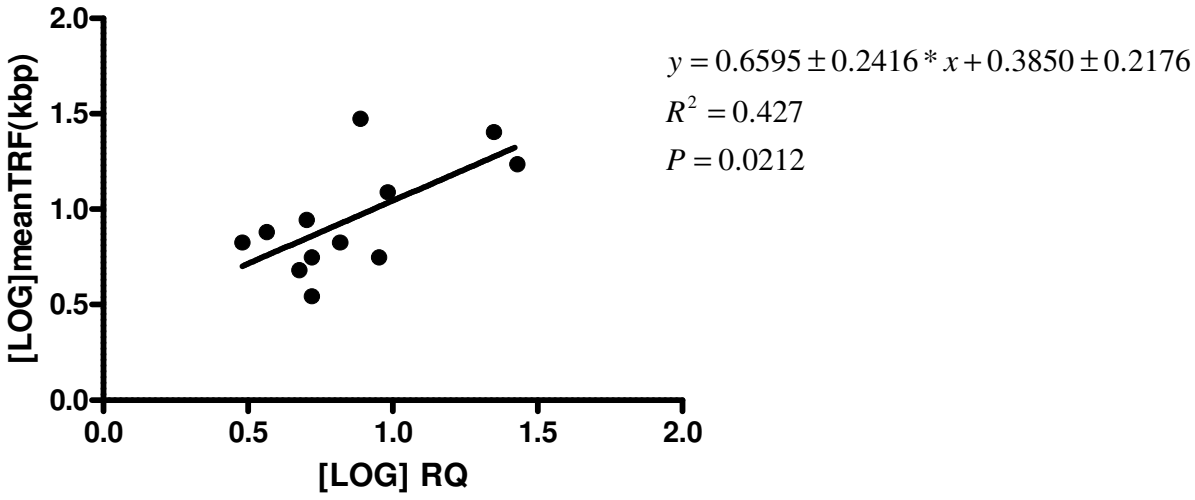


Figure 5.14 **RQs for telomere length obtained with quantitative real-time PCR assay.**
RQ (T/S) values were related to the median of non-tumour tissues (epileptic foci) and were visualized log10 scaled on a grouped scatter blot. Lines within a group of points represent median. Tumour and non-tumour tissue samples are characterized by a filled circle. Astrocytoma cell lines are marked with open circles, Daoy (medulloblastoma) is marked by a black triangle and Saos-2 is marked with an x. Median over all samples is indicated by the dashed line. ** = $p<0.01$ by Mann-Whitney test.

The resulting RQ values from Figure 5.14 and the mean TRF values in kb from 13 tissue samples and 5 cell lines (table 5.1) were compared. Only tissues were tested for linear correlation and GBM was excluded because of problems for quantification in Real time PCR. RQ values are generally not normal distributed (tested by D´Agostino-Pearson, Shapiro-Wilk and Kolmogrov-Smirnov test) and showed no normal distributrion. Both types of values were transformed by decimal logarithm. In this way the values become a normal distribution, and were analysed by Pearson correlation (Figure 5.15). Linear regression analyses results a fitting line: [log] TRF = 0.6595 ± 0.2416x ([log] RQ) + 0.3850 ± 0.2176 with $R^2 = 0.4271$ and P = 0.0212. With this formula, measured RQ values obtained by PCR can be converted in absolute mean TRF values.



Figur 5.15 **Correlation of meanTRF and and RQs of quantitative real-time PCR assay.**
Both, meanTRFs and RQs are log10 transformed before linear correlation by Pearson. A weak but significant correlation between the results of the two different methods is shown. Only tissue samples were tested for linear correlation. GBM27 was excluded because of problems with quantification in Real time PCR).

Next, with the formula the mean TRF values were calculated from all RQ data analysed (Table 5.6 a + b). Calculated TRF values of the groups were summed up by calculation of mean and standard deviation. The absolute telomere lengths based on mean TRF values of the groups (mean±standard deviation in kb) were compared. Telomere lengths increased in both grade II and III tumours DA (21.6 ± 14.9 kb) and AA (17.4 ± 4.5 kb), and in cell lines (18.4 ± 10.7 kb) as compared to GBM (8.8 ± 4.0 kb) and non-tumour tissue (3.1± 2.5 kb).

Tumours acquire an immortal phenotype with a telomere maintenance mechanism (TMM). Telomere length is a criterion to differ between TMM by either the ribonucleoprotein enzyme telomerase or a recombination-based alternative lengthening of telomeres (ALT) mechanism, the latter was found to be a prognostic indicator for patients with glioblastoma multiforme [39]. Criteria for alt are no telomerase activity and a telomere length > 17kb.[40]
In this study results of telomere length measurement show that DA and AA show a mean telomere length >17kb and no telomerase activity. So DA and AA could be ALT, in contrast to GBM, which have a mean telomere length of 8.8 kb and 50% (14 of 28) of them show telomerase activity. Telomerase activity was determined in a former study (diploma thesis of Sibylle Pramhas).

Table 5.6a **Telomere length of tissues**

tissue	#	mean TRF (kb)	RQ	calculated TRF (kb)	mean	SD
GBM	1		8.41	9.9	8.8	4.0
	2	4.8	4.75	6.8		
	3		4.58	6.6		
	4	8.8	5.04	7.1		
	5		8.17	9.7		
	6		7.46	9.1		
	7		19.32	17.1		
	8		6.21	8.1		
	9		0.08	0.5		
	10	17.2	26.96	21.3		
	11	7.6	3.67	5.7		
	12		7.00	8.8		
	13		5.34	7.3		
	14	6.7	3.02	5.0		
	15		7.25	9.0		
	16	3.5	5. 25	7.2		
	17		5.63	7.6		
	18		7.55	9.2		
	19		16.62	15.5		
	20	5.6	8.96	10.3		
	21		8.97	10.3		
	22		10.12	11.2		
	23	12.3	9.60	10.8		
	24		6.55	8.4		
	25	6.7	6.59	8.4		
	26		4.09	6.1		
	27	9.2	1.33	2.9		
	28	5.6	5.25	7.2		
AA	1	25.4	22.33	18.8	17.4	4.5
	2		16.21	15.2		
	3		8.90	10.3		
	4		22.17	18.7		
	5		20.67	17.9		
	6		31.6	23.7		
DA	1		77.68	42.8	21.6	14.9
	2		19.00	16.9		
	3		20.83	18.0		
	4		5.55	7.5		
	5		0.36	1.2		

tissue	#	mean TRF (kb)	RQ	calculated TRF (kb)	mean	SD
	6		82.34	44.5		
	7		44.04	29.5		
	8		10.70	11.6		
	9		44.73	29.8		
	10	29.8	7.74	9.4		
	11		8.15	9.7		
	12		66.05	38.5		
HC	1		1.34	2.9	3.1	2.5
	2		0.45	1.4		
CO	1		0.66	1.8		
	2		0.22	0.9		
TM	1		5.86	7.8		
	2		1.71	3.5		

GBM glioblastoma multiforme (grade IV)
AA anaplastisc astrocytoma (grade III)
DA diffuse astrocytoma (grade II)
HC Hippocampus
CO Cortex
TM temporal mark
* non-tumour tissues are taken from these brain regions from two patients

Table5.6b **Telomere length of cell lines**

tissue	cell line	mean TRF (kb)	RQ	calculated TRF (kb)	mean	SD
Astrocytoma	CCF-STTG1		n.a.	n.a	18.4	10.7
	DBTRG/05MG		26.60	21.1		
	KG-MH		20.44	17.8		
	KM-YH	4.8	34.46	25.1		
	LN-140		23.11	19.3		
	MGC		1.04	2.5		
	MR1	2.5	9.60	10.8		
	T98G		10.96	11.8		
	U-373 MG		16.52	15.4		
	YT-BO	8.8	83.69	45.0		
	YU-PM		11.37	12.1		
	Hs683					
Medulloblastoma	Daoy		13.86	13.7		
Osteosarcoma	SAOS-2	8.9	37.46	26.5		

Tables 5.6b show the meanTRF length in kb (determined on Southern blots) and the RQs from the quantitative real-time PCR assay of cell lines. Additionally, mean TRFs were calculated by interpolation with a formula as described in the text.

5.3 Real-time PCR assay for measuring TERRA expression

Overall telomere expression was determined by the same PCR assay as for telomere length measurement. Reverse transcribed RNA termed as cDNA was used instead of genomic DNA for expression analysis of telomeric repeats.

In contrast to the assay for amplifying the telomere repeats for overall TERRA, I established assays with specific primers from the subtelomeric region for the analyses of TERRA from chromosomal ends 2p and 18p. The calculated and expected length for the amplicons for 2p TERRA and 18p TERRA are 109 bp and 149 bp, respectively.
Primer sequences were tested in CloneManager9 by aligning them against the genomic sequences of subtelomeric regions of chromosome 2p and 18p (Figure 5.16 and 5.17, respectively). Genomic sequences were taken from MapViewer Homo sapiens, build 37.1.

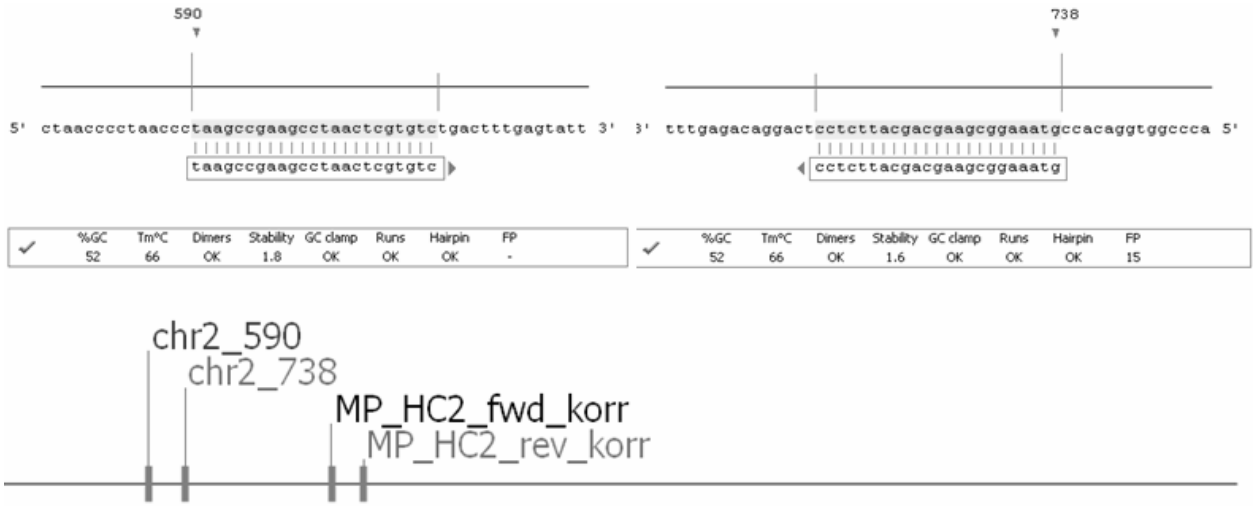


Figure 5.16 **Aligning of primers chr2_590 and chr2_738 to the genomic sequence of subtelomeric region of chromosome 2p.** Results of the primer test with CloneManager 9 program is presented. Lower panel: line represents 5kbp of sub-telomeric sequence with the telomere positioned on the right (5') site, primers for expression of 2pTERRA and for methylation-specific PCR of subtelomeric region of chromosome 2p are shown, note: the hypothetical transcription start site is located between both amplicons.

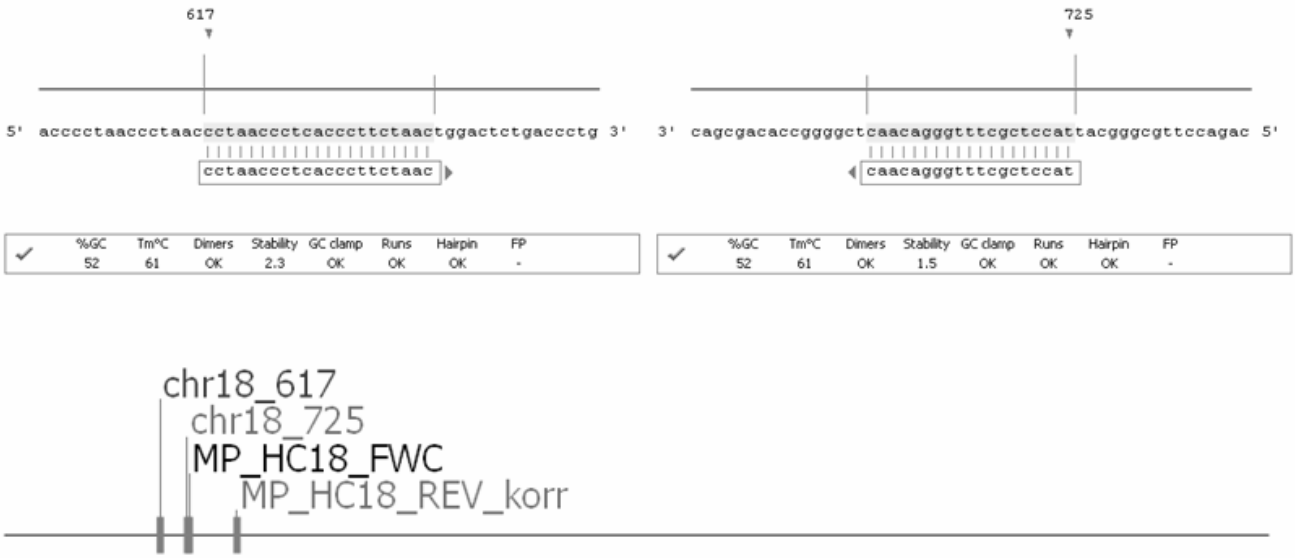


Figure 5.17 **Aligning of primers chr18_617 and chr18_725 to the genomic sequence of subtelomeric region of chromosome 18p** in CloneManager 9 program. Lower panel: line represents 5kbp of sub-telomeric sequence with the telomere positioned on the right (5') site, primers for expression of 2pTERRA and for methylation-specific PCR of subtelomeric region of chromosome 2p are shown, note: the hypothetical transcription start site is located between both amplicons.

Primers were tested by real-time PCR, and amplicons were analysed by PAGE (Figure 5.18 and 5.19). Amplicons showed the expected length of 149 and 109 bp, respectively. Both NTC controls gave no signal during amplification and on the temperature melting profile and consequently on the gel.

(a) (b)

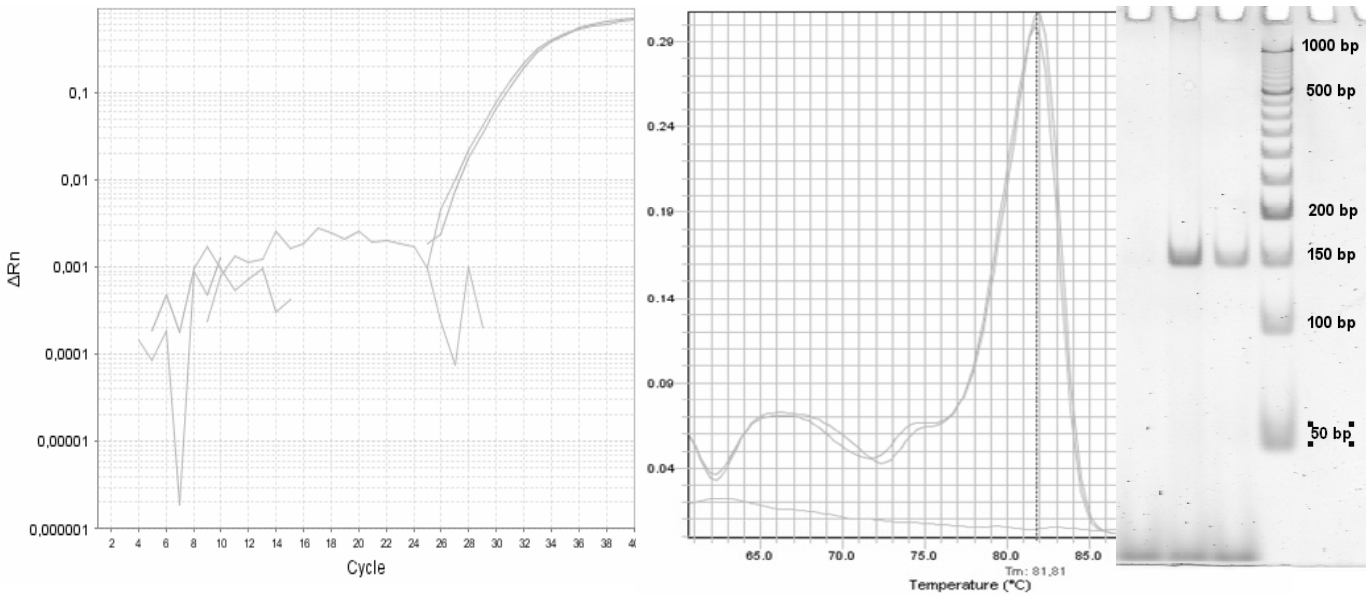


Figure 5.18 (a+b) 2p TERRA

(a) shows the amplification of 2p TERRA in an astrocytoma tissue sample, and

(b) shows the melting curve of the PCR product; the amplicon was also loaded on a PAGE-gel to show the specifity of the assay: non-template control (lane 1), amplicon from genomic DNA (lane 2) and from reverse transcribed DNA (lane 3).

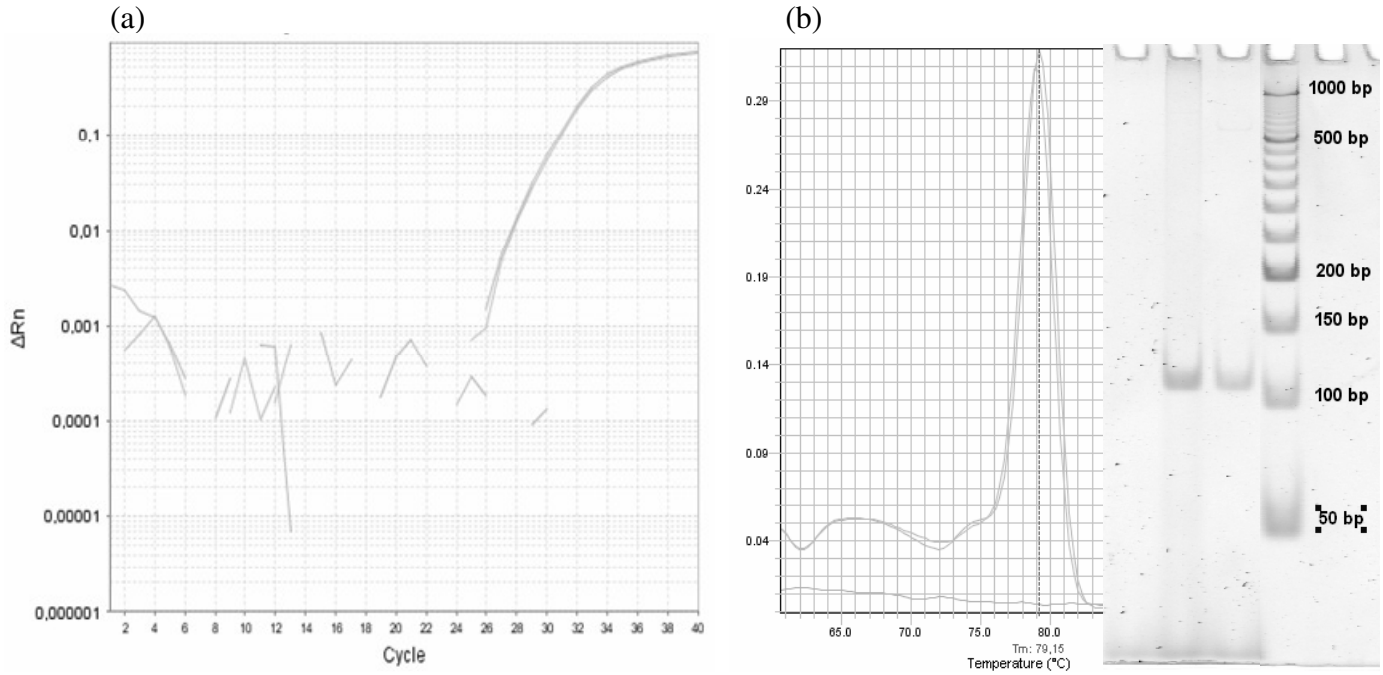


Figure 5.19 (a+b) 18p TERRA

(a) shows the amplification of 18p TERRA in an astrocytoma tissue sample, and

(b) shows the melting curve of the PCR product; the amplicon was also loaded on a PAGE-gel to show the specifity of the assay: non-template control (lane 1), amplicon from genomic DNA (lane 2) and from reverse transcribed DNA (lane 3).

RQ values obtained with this assays, were determined relative to median of non-tumour tissues and to expression levels of ribosomal gene 36B4 (Table 5.5 a + b). If 2p or 18pTERRA was not detected in real-time PCR, a Ct of 35 was assumed to calculate the RQ for these samples.

Experiments for overall TERRA and 2pTERRA and 18pTERRA run in 7500 Fast Real-Time PCR system (Applied Biosystems) and to analyse results, they were combined in a study in ABI SDS Software version2.2.

For overall TERRA, 6 96-well plates were combined in a study. 7 wells showed high SD, 8 wells showed no amplification and 5 were marked as ouliers in replicate groups (Table 5.10)

For 2p TERRA and 18pTERRA, 17 96-well plates were in the study. 56 wells showed no amplification of the sample. In 32 wells the software was not able of identify an exponential region of amplification. 23 wells were marked for high SD (Table 5.11).

By comparison of mean Ct of the two housekeeping genes, it can be seen, that in allmost all samples tested (cell lines and tissues), 36B4 is 2 – 3 Ct earlier compared to b-Actin (Table 5.7a+b).

Table 5.7a **Comparison of two house keeping genes in cell lines**

tissue	cell line	36B4*	b-Actin*
Astrocytoma	CCF-STTG1	15.2	16.5
	DBTRG/05MG	16.0	17.8
	KG-MH	14.3	16.4
	KM-YH	15.7	17.1
	LN-140	14.6	15.2
	MGC	14.4	16.3
	MR1	15.7	16.4
	T98G	14.9	16.4
	U-373 MG	15.6	17.3
	YT-BO	14.8	16.7
	YU-PM	14.1	17.5
	Hs683	14.3	17.1
Medulloblastoma	Daoy	14.5	17.2
Osteosarcoma	SAOS-2	14.7	17.5

*mean Ct for 36B4 and b-Actin are given

Table 5.7b **Comparison of two house keeping genes in tissues**

tissue	#	36B4*	b-Actin*
GBM	1	15.5	19.4
	2	14.1	16.9
	3	15.5	17.9
	4	16.3	18.0
	5	18.2	19.9
	6	16.9	19.1
	7	15.1	17.2
	8	15.7	17.9
	9	16.5	18.8
	10	15.8	17.8
	11	15.4	18.2
	12	15.9	18.9
	13		
	14	16.0	19.4
	15	16.5	18.7
	16	16.4	19.1
	17	15.6	17.8
	18	16.1	18.7
	19	17.6	19.7
	20	14.8	17.4
	21	14.2	16.5
	22	15.6	18.0
	23	15.6	17.6
	24	14.5	18.1
	25	15.2	18.1
	26	16.2	19.4
	27	18.1	18.9
	28	15.7	17.9
AA	1	15.8	17.9
	2	17.2	19.1
	3	17.2	18.2
	4	19.6	21.2
	5	18.1	20.1
	6	18.4	18.7
DA	1	17.5	18.2
	2	18.3	19.4
	3	16.0	17.0
	4	17.3	18.7
	5	17.4	18.7
	6	17.4	19.2
	7	17.8	19.1
	8	16.8	19.3
	9	16.8	19.1
	10	15.5	18.8
	11	17.2	18.3
	12	15.6	17.7
HC	1	17.6	20.8
	2	16.2	20.7
CO	1	17.2	21.5
	2	16.1	20.2
TM	1	18.2	21.8
	2	17.6	20.8

*mean Ct for 36B4 and b-Actin are given

Table 5.8a **Overall, 2p and 18pTERRA expression in tissue samples**

		compared to 36B4			compared to beta-actin	
tissue	#	overall	chr.2p	chr.18p	chr.2p	chr.18p
GBM	1	0.0018	0.0637	0.3223	0.0407	0.2052
	2	0.0004	0.0259	0.0626	0.0360	0.0867
	3	0.0091	0.1404	0.6851	0.2467	1.1981
	4	0.0028	0.1682	0.1785	0.4988	0.5269
	5	0.0299	1.1464	0.6823	3.4295	2.0324
	6	0.0010	0.1984	0.2333	0.4139	0.4846
	7	0.0007	0.0351	0.0309	0.0780	0.0684
	8	0.0316	0.0896	0.2916	0.1854	0.6008
	9	0.0007	0.0235	0.2391	0.0463	0.4685
	10	0.3971	1.2572	3.1678	2.9823	7.4822
	11	0.0038	0.1285	0.2961	0.1843	0.4229
	12	0.0043	0.3358	0.0066	0.4315	0.0084
	13	0.0108	1.2479	3.0662	1.2558	5.2062
	14	0.0028	0.1195	0.0651	0.1118	0.0607
	15	0.0045	0.3632	0.0026	0.7353	0.0053
	16	0.0003	0.0807	0.0055	0.1143	0.0078
	17	0.0040	0.2383	0.0323	0.5246	0.0708
	18	0.0080	0.4967	0.3773	0.8001	0.6051
	19	0.0065	0.5540	0.2768	1.1721	0.5832
	20	0.0043	0.2395	0.3508	0.3644	0.5315
	21	0.0002	0.0331	0.1490	0.0640	0.2871
	22	0.0030	0.0463	0.4562	0.0825	0.8099
	23	0.0025	0.0437	0.0627	0.1046	0.1494
	24	0.0005	0.0241	0.0873	0.0195	0.0704
	25	0.0004	0.3415	1.7163	0.4477	2.2402
	26	0.0034	0.1676	1.0869	0.1773	1.1451
	27	0.0022	0.3346	0.1429	1.9212	0.8170
	28	0.0867	0.1801	0.0356	0.3619	0.0713
AA	1	0.0137	0.0436	0.0416	0.0958	0.0911
	2	0.2444	0.3075	2.2598	0.7956	5.8207
	3	0.0027	0.0894	0.5318	0.4560	2.7016
	4	0.1081	0.2245	1.8099	0.7085	5.6863
	5	0.0060	1.0708	0.0740	2.6237	0.1805
	6	0.0751	0.1750	0.9934	1.2900	7.2930
DA	1	0.4666	0.4087	0.1403	2.4263	0.8290
	2	0.0465	0.1205	0.0919	0.5488	0.4165
	3	0.0488	0.1067	0.1020	0.5076	0.4830
	4	0.3968	0.3341	0.0653	1.1545	0.2338
	5	0.1140	0.2369	0.1658	0.9186	0.6403
	6	0.1251	0.8444	0.0604	2.3520	0.1674
	7	0.0266	0.1369	0.6285	0.5185	2.3700
	8	0.0004	0.4083	0.1604	0.6555	0.2564
	9	0.1549	0.1006	0.4152	0.1985	0.8155
	10	0.1120	0.7170	2.4352	0.7061	2.3880
	11	0.0274	0.7585	1.4806	3.3682	6.5464
	12	0.0862	0.0413	0.3637	0.0946	0.8290
HC	1	0.8806	0.5457	0.7815	0.5603	0.7989
	2	0.4405	5.0213	3.0735	2.0399	1.2433
CO	1	3.8261	0.2920	0.0844	0.1415	0.0407
	2	1.4357	0.2350	0.0910	0.1348	0.0519
TM	1	0.2989	6.4059	26.7621	5.0147	20.8595
	2	1.1194	1.4543	1.2185	1.4397	1.2011

Table 5.8b **Overall, 2p and 18p TERRA expression in cell lines**

tissue	cell line	overall	chr.2p	chr.18p	chr.2p	chr.18p
		relative to 36B4			relative to b-actin	
Astrocytoma	CCF-STTG1	0.0041	0.0022	0.0350	0.0061	0.3307
	DBTRG/05MG	0.0179	0.0172	0.0191	0.0372	0.0412
	KG-MH	0.0075	0.0095	0.1347	0.0343	0.4868
	KM-YH	0.0092	0.0023	0.0128	0.0217	0.0782
	LN-140	0.0030	0.0117	0.0124	0.0297	0.1169
	MGC	0.0239	0.0062	0.0103	0.0354	0.0590
	MR1	0.0036	0.0053	0.0068	0.0499	0.0223
	T98G	0.0037	0.0218	0.0247	0.0672	0.2338
	U-373 MG	0.0021	0.0007	0.0163	0.0017	0.1543
	YT-BO	0.0088	0.0153	0.0105	0.0143	0.0097
	YU-PM	0.0327	0.0410	0.0409	0.0583	0.0578
	Hs683	0.0319	0.0200	0.0250	0.0282	0.0350
Medulloblastoma	Daoy	0.0186	0.0697	0.0775	0.2727	0.3017
Osteosarcoma	SAOS-2	0.2107	0.1134	0.0262	0.1577	0.0362

Table 5.8a and 5.8b show RQ-values of expression of overall TERRA, 2pTERRA and 18pTERRA. RQs for overall TERRA are relative to 36B4; 2pTERRA and 18pTERRA are relative to 36B4 and b-actin.

If 2p or 18pTERRA was not detected in Real-time PCR, a Ct of 35 was assumed to calculate the RQ for these samples.

GBM glioblastoma multiforme (grade IV)

AA anaplastisc astrocytoma (grade III)

DA diffuse astrocytoma (grade II)

HC Hippocampus*

CO Cortex*

TM temporal mark*

* non-tumour tissue is taken from these brain regions from two patients

RQ values (related to 36B4) were presented grouped on a scatter blot with log10 scale (Figure 5.20 (a-c). Overall TERRA declined gradually from non-tumour tissues over DA, AA to GBM. Overall TERRA expression was decreased 14 and 31-fold in GBM compared to AA and DA ($p<0.05$), respectively. 2p TERRA showed the same trend like overall TERRA to decline gradually, whereas 18p TERRA did not show to follow this trend.

From all cell lines analysed, SAOS-2 showed for 2p TERRA and overall TERRA the highest expression level. But not for 18p TERRA, the expression level was comparable to the other cell lines analysed.

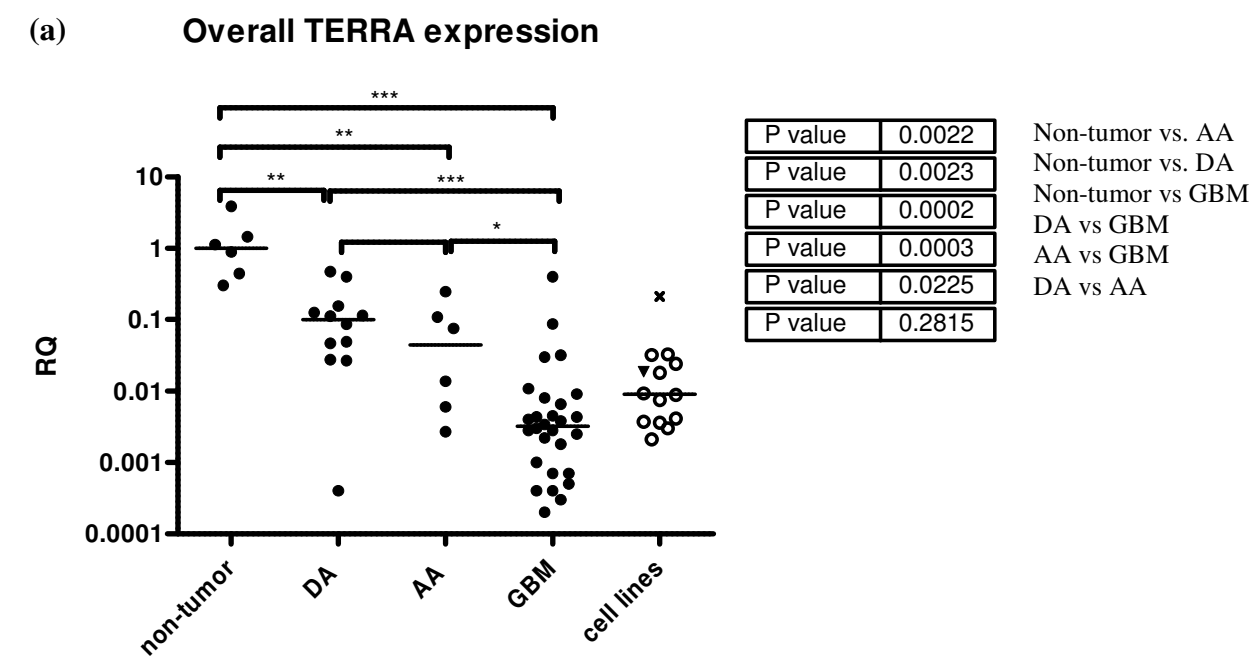


Figure 5.20 (a) **Expression of overall TERRA in brain tissue samples and astrocytoma cell lines and also Saos-2. RQ related to 36B4.**

(a) Overall TERRA shows a decrease over grades from non-tumour tissue, over low grade tumour samples to glioblastomas multiforme. Saos-2 shows the highest overall TERRA expression in cell lines.

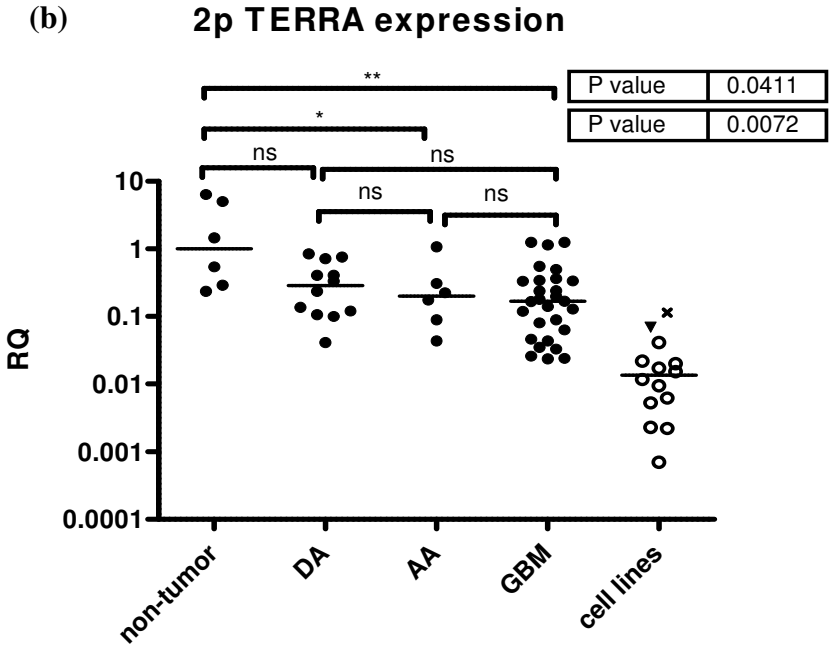


Figure 5.20 (b) **Expression of 2pTERRA** in brain tissue samples and astrocytoma cell lines and also Saos-2. RQ related to 36B4.

Expression of 2p TERRA: Comparable to overall TERRA expression, 2p TERRA expression is declining with the grade of tumour. Non-tumour tissue shows the highest expression of 2p TERRA- Saos-2 shows the highest level on 2p TERRA expression for cell lines.

c)

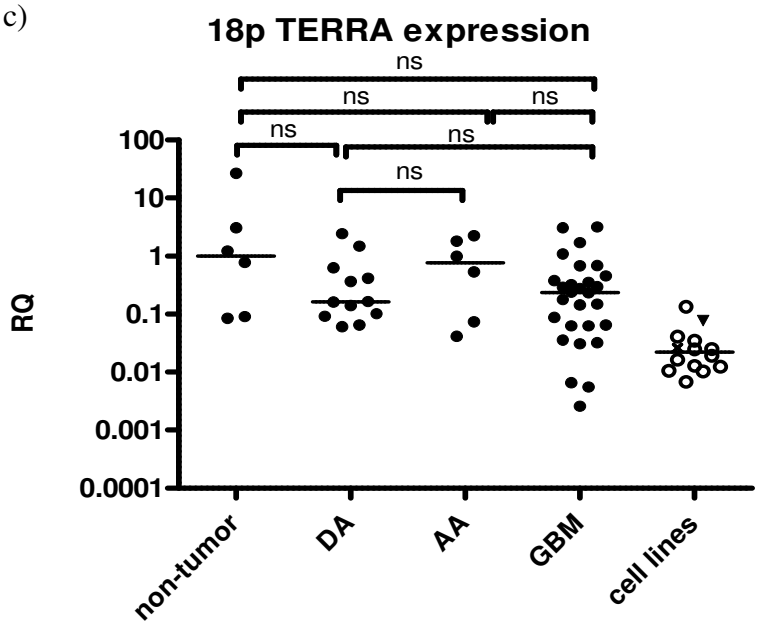


Figure 5.20 (c) **Expression of 2pTERRA** in brain tissue samples and astrocytoma cell lines and also Saos-2. RQ related to 36B4.
18p TERRA shows no correlation with grade. Brain tumour cell lines are labelled with open circles, whereas Saos-2 is shown by an x.

Linear correlation performed on log transformed data of the astrocytic brain tumour samples revealed a link between TERRA expression and telomere length. Overall TERRA levels correlated weak but significantly with TERRA of 2p (Pearson r = 0.5251value = 0.0021) and 18p (Pearson r = 0.3407 P value = 0.0134) chromosomal ends and with RQ of telomere length (Pearson r = 0.4052, P value = 0.0006) as shown in Figure 5.21 a – c.

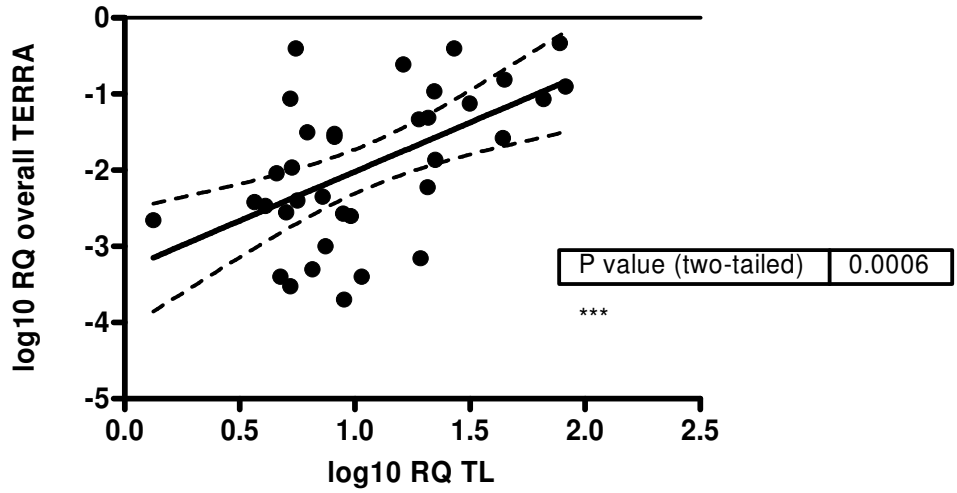


Figure 5.21(a) **Correlation test and linear regression between telomere length and expression levels of overall TERRA** in astrocytoma tissue samples.

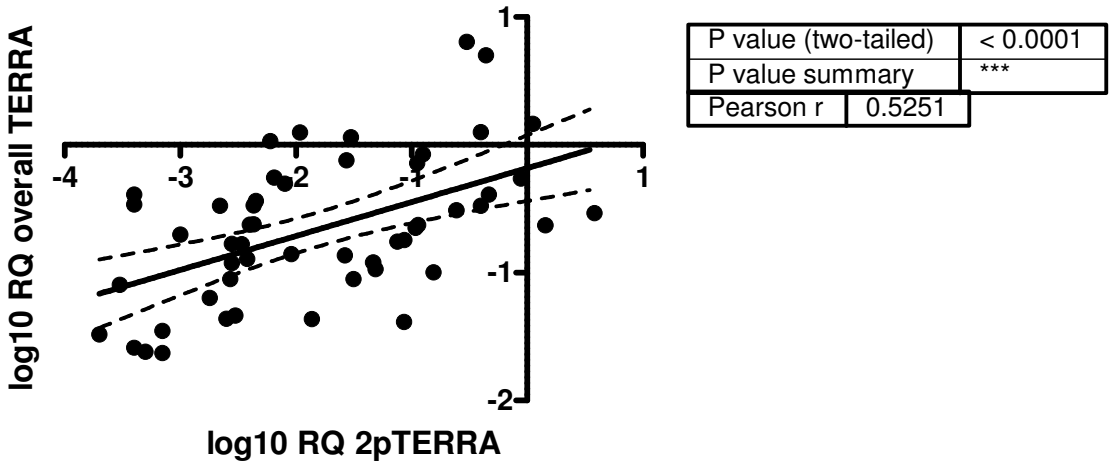


Figure 5.21(b) Correlation of 2pTERRA with overall TERRA (only tissues are used for correlation)

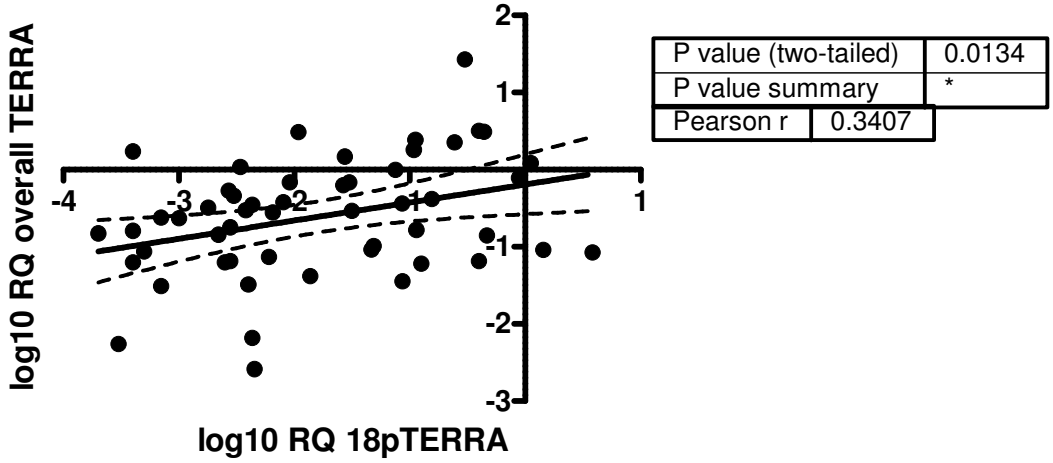


Figure 5.21(c) **Correlation of 18p TERRA with overall TERRA** (only tissues are used for correlation)

To demonstrate the specifity of the new designed assays for 2pTERRA and 18pTERRA, cDNA of one GBM tumour tissue was PCR amplified and PCR products where cloned in pCRII TOPO vector by TOPO TA cloning.

Plasmids were isolated from 3 blue clones (supposed to contain inserts) from 2pTERRA and 18pTERRA products with WIZARD® Plus SV Miniprep DNA Purification System (described in Materials and Methods, chapter 4.5). Plasmids were analyzed by restriction with *Eco*RI to cut out the cloned insert from the vector (Figure 5.22a).

For a second test, isolated plasmids were amplified by PCR with primers chr2_590 + chr2_738 and chr18_617 + chr18_725. Two of the isolated clones of 2pTERRA and all 3 clones of 18pTERRA did show the expected insert size, and only one clone of 2pTERRA did not or failed PCR amplification (Figure5.22b).

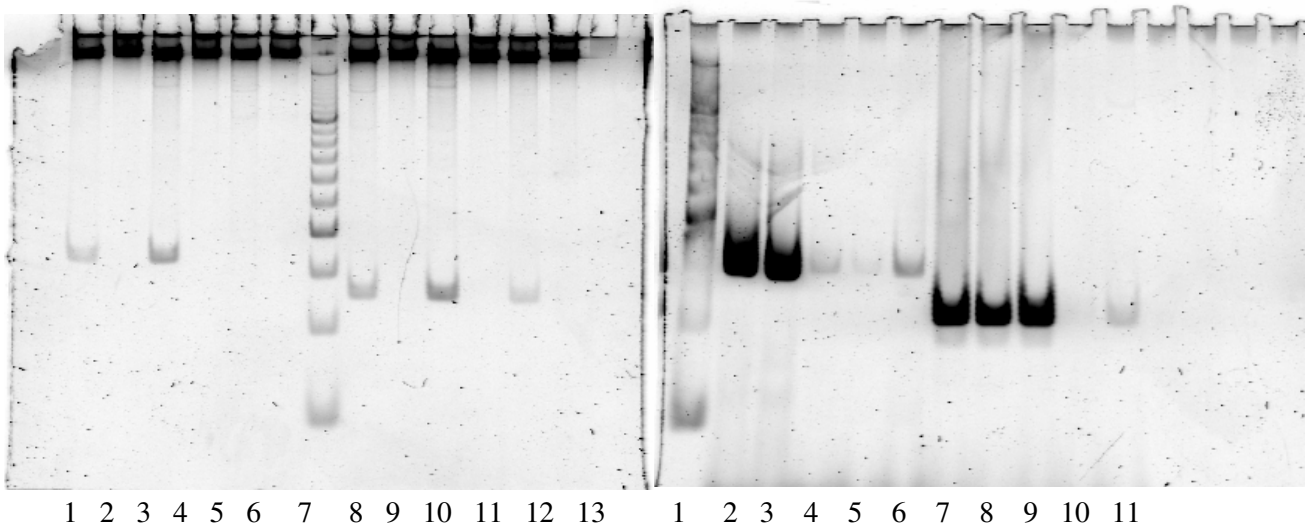


Figure 5.22 (a+b) **Agarose gel for analysing clones of 2pTERRA and 18pTERRA**

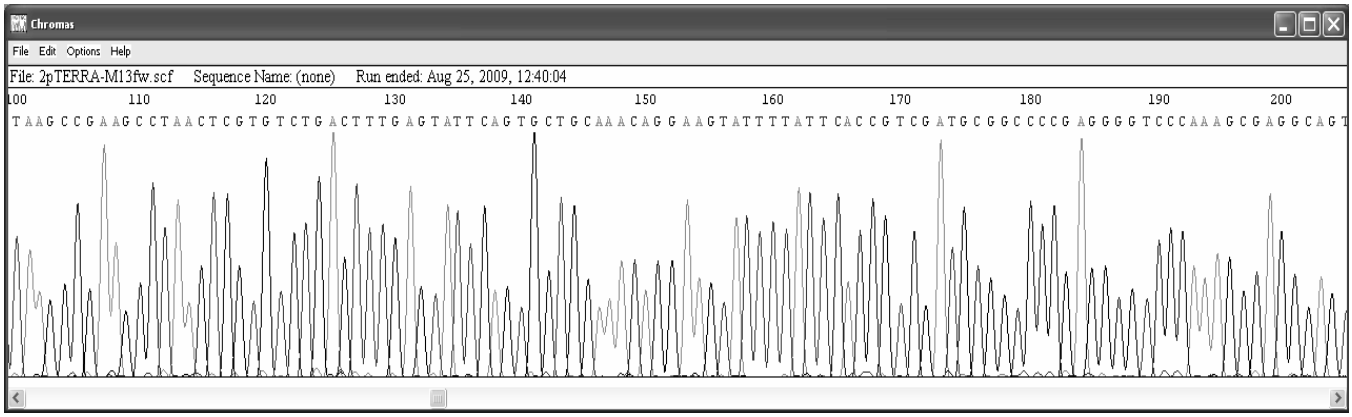
(a) Plasmids were digested with EcoRI and loaded on a 5% PAGE-gel; 2p_clone1digested (lane1); 2p_clone1undigested (lane2); 2p_clone2 digested (lane3); 2p_clone2 undigested (lane4); 2p_clone3 digested (lane5); 2p_clone3 undigested (lane6); 50bp Marker (lane 7); 18p_clone1digested (lane8); 18p_clone1undigested (lane9); 18p_clone2 digested (lane 10); 18p_clone2 undigested (lane 11); 18p_clone3 digested (lane 12); 18p_clone3 undigested (lane 13);

(b) Plasmids are PCR amplified with primers chr2_590 and chr2_738 for clones of 2pTERRA and chr18_617 and chr18_750 for clones of 18pTERRA; 50bp Marker (lane1); 2p_clone 1 (lane 2); 2p_clone2 (lane 3); 2p_clone3 (lane 4); NTC_primers chr2_590 and chr2_738 (lane 5); 18p_clone 1 (lane 6); 18p_clone2 (lane 7); 18p_clone3 (lane 8); NTC_primers chr18_617 and chr18_750 (lane 9);

Only 2p_Klon3 shows not the correct insert, all other plasmid carry the expected insert.

One clone of 2pTERRA and one clone from 18pTERRA were sequenced by VBC genomics, and the obtained sequences where aligned in Clonemanager 9 program against the sequences of the subtelomeric region of chromosome 2p and 18p, respectively (Figure 5.23). The sequences were 100 percent identical.

Genomic sequence for the alignment was taken from MapViewer homo sapiens, build 37.1 for both subtelomeric regions (chromosome 2p and 18p).



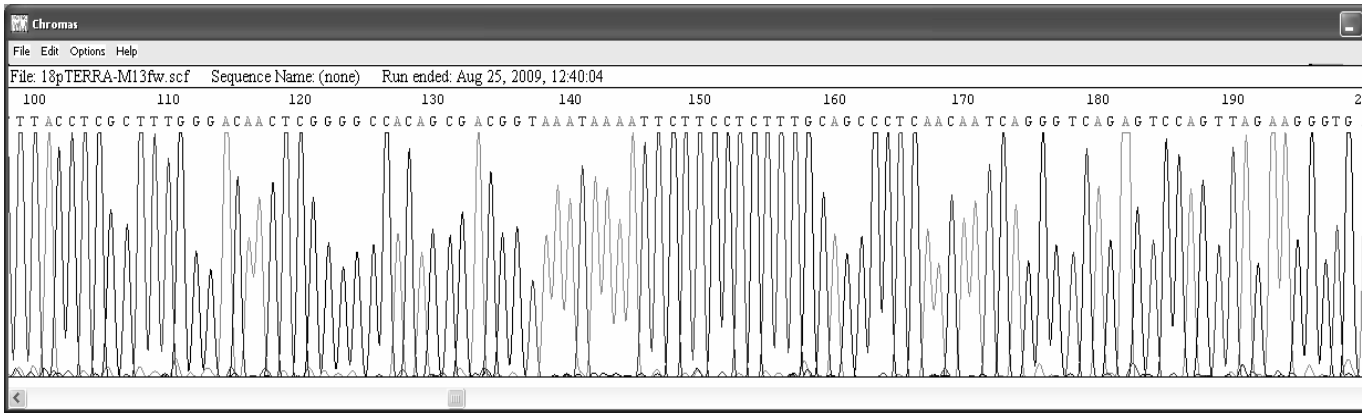

```
NoName                1 cagtgaattgtaatacgactcactatagggcgaattgggccctctagatgcatgctcgag
chrom2 (590-738)        ------------------------------------------------------------

NoName               61 cggccgccagtgtgatggatatctgcagaattcgcccttaagccgaagcctaactcgtg
chrom2 (590-738)      1 ----------------------------------------taagccgaagcctaactcgtg

NoName              121 tctgactttgagtattcagtgctgcaaacaggaagtattttattcaccgtcgatgcggcc
chrom2 (590-738)     22 tctgactttgagtattcagtgctgcaaacaggaagtattttattcaccgtcgatgcggcc

NoName              181 ccgaggggtcccaaagcgaggcagtgcccccaaactctgtcctgaggagaatgctgcttc
chrom2 (590-738)     82 ccgaggggtcccaaagcgaggcagtgcccccaaactctgtcctgaggagaatgctgcttc

NoName              241 gcctttacaagggcgaattccagcacactggcggccgttactagtggatccgagctcggt
chrom2 (590-738)    142 gcctttac------------------------------------------------------

NoName              301 accaagcttgatgcatagcttgagtattctatagtgtcacctaaatagcttggcgtaatc
chrom2 (590-738)        ------------------------------------------------------------

NoName              361 atggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacg
chrom2 (590-738)        ------------------------------------------------------------

NoName              421 a
chrom2 (590-738)        -
```

Figure 5.23a **Sequencing results for 2pTERRA.** Sequence similarity was tested in Clone Manager 9 by DNA alignment. Genomic sequence was taken from MapViewer homo sapiens, build 37.1

Non-matching bases are coloured

Top panel … part of sequencing result from VBC Genomic (nt100 – 200, nt100 is start of the 2pTERRA sequence, as can be seen in the button panel, sequenes start to be identical)

Button panel … alignment of genomic sequence (nt 590 – 738) shows sequences are 100% identical




```
chrom18 (617-725          ------------------------------------------------------------
NoName                421 ctcattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtggaattg

chrom18 (617-725          ------------------------------------------------------------
NoName                361 tgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaagctattta

chrom18 (617-725          ------------------------------------------------------------
NoName                301 ggtgacactatagaatactcaagctatgcatcaagcttggtaccgagctcggatccacta

chrom18 (617-725            1 ----------------------------------cctaaccctcacccttctaactggact
NoName                241 gtaacggccgccagtgtgctggaattcgcccttcctaaccctcacccttctaactggact

chrom18 (617-725           28 ctgaccctgattgttgagggctgcaaagaggaagaattttatttaccgtcgctgtggccc
NoName                181 ctgaccctgattgttgagggctgcaaagaggaagaattttatttaccgtcgctgtggccc

chrom18 (617-725           88 cgagttgtcccaaagcgaggta--------------------------------------
NoName                121 cgagttgtcccaaagcgaggtaaagggcgaattctgcagatatccatcacactggcggcc

chrom18 (617-725          ------------------------------------------------------------
NoName                 61 gctcgagcatgcatctagagggcccaattcgccctatagtgagtcgtattacaatcactg

chrom18 (617-725          -
NoName                  1 c
```

Figure 5.23b **Sequencing results for 2pTERRA.** Sequence similarity was tested in Clone Manager 9 by DNA alignment. Genomic sequence was taken from MapViewer homo sapiens, build 37.1

Non-matching bases are coloured

Top panel … part of sequencing result from VBC Genomic (nt100 – 200, nt100 is start of the 18pTERRA sequence, as can be seen in the button panel, sequenes start to be identical)

Button panel … alignment of genomic sequence (nt 617 – 725) shows sequences are 100% identical

Table 5.10 **QC-Summary of overall TERRA study**

Biological Replicate Reference Sample	Epi
Chemistry	SYBR® Reagents
Created By	SS
Description	
Endogenous Control(s)	36B4
Instrument Type(s)	sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast
Last Modified	Wed May 27 18:04:33 CEST 2009
Number of Experiments	6
RQ Confidence Level	95.0
Reaction Volume	8.0
Study Name	Overall TERRA expression in astrocytic brain tumours
Study Type	RQ
Technical Replicate Reference Sample	gbm10

Flag	Name	Description	Criteria	Frequency
SPIKE	Noise spikes	The amplification plot contains one or more data points inconsistent with the other points in the plot	Spike algorithm result > 1.0	1
BADROX	Bad passive reference signal	Passive reference signal is abnormal	Bad passive reference algorithm result > 0.6	0
NOAMP	No amplification	The sample did not amplify	Amplification algorithm result < 0.1	8
CTFAIL	C_T algorithm failed	The software cannot calculate C_T.		0
BLFAIL	Baseline algorithm failed	The software cannot calculate the best fit baseline for the data.		0
EXPFAIL	Exponential algorithm failed	The software cannot identify the exponential region of the amplification plot.		2
THOLDFAIL	Thresholding algorithm failed	The software cannot calculate a threshold.		0
HIGHSD	High standard deviation in replicate group	Ct standard deviation for the replicate group exceeds the flag setting.	Ct standard deviation > 0.5	7
OUTLIERRG	Outlier in replicate group	Ct deviates significantly from Ct values in the associated replicate group		5
NOSIGNAL	No signal in well	The well produced very low or no fluorescence.		0
NOISE	Noise higher than others in plate	The well produced more noise in the amplification plot than other wells in the plate	Relative noise > 4.0	7
OFFSCALE	Fluorescence is offscale	Fluorescence exceeds the instrument's maximum detectable range for one or more cycles.		0
AMPNC	Amplification in negative control	A sequence amplified in a negative control reaction.	C_T < 35.0	0

Table5.11 **QC summary for 2p and 18p TERRA expression study**

Chemistry	Benign
Created By	SYBR® Reagents
Description	SS
Endogenous Control(s)	betaaktin, 36B4
Instrument Type(s)	sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast, sds7500fast
Last Modified	Fri Jun 26 18:02:06 CEST 2009
Number of Experiments	17
RQ Confidence Level	95.0
Reaction Volume	8.0
Study Name	2p and 18p TERRA expression in astrocytic brain tumours
Study Type	RQ
Technical Replicate Reference Sample	GBM10

Flag	Name	Description	Criteria	Frequency
SPIKE	Noise spikes	The amplification plot contains one or more data points inconsistent with the other points in the plot	Spike algorithm result > 1.0	9
BADROX	Bad passive reference signal	Passive reference signal is abnormal		0
NOAMP	No amplification	The sample did not amplify	Amplification algorithm result < 0.1	56
CTFAIL	C_T algorithm failed	The software cannot calculate C_T.		0
BLFAIL	Baseline algorithm failed	The software cannot calculate the best fit baseline for the data.		0
EXPFAIL	Exponential algorithm failed	The software cannot identify the exponential region of the amplification plot.		32
THOLDFAIL	Thresholding algorithm failed	The software cannot calculate a threshold.		0
HIGHSD	High standard deviation in replicate group	Ct standard deviation for the replicate group exceeds the flag setting.	Ct standard deviation > 0.5	23
OUTLIERRG	Outlier in replicate group	Ct deviates significantly from Ct values in the associated replicate group		2
NOSIGNAL	No signal in well	The well produced very low or no fluorescence.		0
NOISE	Noise higher than others in plate	The well produced more noise in the amplification plot than other wells in the plate	Relative noise > 4.0	9
OFFSCALE	Fluorescence is offscale	Fluorescence exceeds the instrument's maximum detectable range for one or more cycles.		0
AMPNC	Amplification in negative control	A sequence amplified in a negative control reaction.	C_T < 35.0	0

5.4 Subtelomeric methylation and telomeric transcription

Methylation of CpG islands for specific subtelomeric regions were determined by bisulphite allelic sequencing. Genomic DNA was treated with bisulfit for convertion of non-methylated C's into T's and PCR was performed on 100ng of bisulfite converted DNA using primers and conditions as described in Table 4.5 and Table 4.9, respectively. Amplicons were analysed by quantitative sequencing to determine the C/T ratios. A selected subset of high grade tumours with high and low 2p TERRA expression and low grade tumours with high and low 2p TERRA expression was analysed. Primers available for PCR of CpG islands are positioned 1,400 bp from the chromosomal 2p end and 800 bp from the chromosomal 18p end.
DNA samples isolated from GBM27 with both the Trizol method and the Maxwell tissue DNA purification kit were converted and amplified by PCR, purified and send to Qiagen for quantitative sequencing to determine the ratios of non-converted C's which correspond to methylated C's. The methylation status of the CpG islands from the subtelomeric regions was determined of chromosome end 2p and 18p containing 13 and 14 CpG-positions, respectively (Figure 5.24 and Table 5.12). The ratios of methylated to non-methylated CpG positions analysed did not show differences larger than the variation limit of the method (5%).
The methylation of CpG positions varies for CpG island of chromosome 2p between 42 and 100% and demonstrates 90.8% mean methylation. For CpG island of chromosome 18p, methylation of CpG positions vary between 45 and 99% and demonstrate 95.8 mean methylation.

The information from both sequencings should be identical, but was sometimes missing (in case of Maxwell-DNA) or partially available due to problems with the sequencing reaction.

Sequnencing is done by Sanger method so that the Sequencing primers are already bound to the fragment, and that the first bases immediately after the sequencing primer can not be completely dissolved (peaks can not be separated). Therefore, the first ~ 45 bases (25 by the primer, again about 20 because of weak Separation) from any direction are "lost".

Table5.12 **Sequencing results of bislulfite converted DNA** (isolated form Trizol and Chaps lysates)

a)

low 2p TERRA	RQ 2pTERRA	1	2	3	4	5	6	7	8	9	10	11	12	13	mean+SD	median
T_for	0.3346	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	42	96	87	86	96	83	94	83.4 ± 17.6	87
T_rev		96	97	98*	99	97	91*	80	95	n.a.	n.a.	n.a.	n.a.	n.a.	94.1 ± 5.8	96.5
C_for		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	44	98	83	79	100	83	100	83.9 ± 18.3	83
C_rev		98*	97	99	99	95	91*	83	97	n.a.	n.a.	n.a.	n.a.	n.a.	94.9 ± 5.1	97

DNA isolated from GBM27 was isolated by two different methods (Trizol and Chaps remainders) and bisulfite converted.

T_for … DNA isolated from Trizol and sequenced with primer MP_HC2 FWD (table 4.4)

T_rev … DNA isolated from Trizol and sequenced with primer MP_HC2 REV (table 4.4)

C_for … DNA isolated from Chaps and sequenced with primer MP_HC2 FWD (table 4.4)

C_rev … DNA isolated from Chaps and sequenced with primer MP_HC2 REV (table 4.4)

b)

Low18p TERRA	RQ 18pTERRA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	mean+SD	median
T_for	0.1429																
T_rev		45	78	96	95	83	98	97	99	95	90	n.a.	n.a.	n.a.	n.a.	87.6 ± 15.6	95
C_for		n.a.	n.a.	n.a.	n.a.	n.a.	73	98	98	98	70	66	97	99	98	88.6 ± 13.5	98
C_rev		51	94	94	95	82	96	96	98	99	86	n.a.	n.a.	n.a.	n.a.	89.1 ± 13.7	94.5

DNA isolated from GBM27 was isolated by two different methods (Trizol and Chaps remainders) and bisulfite converted.

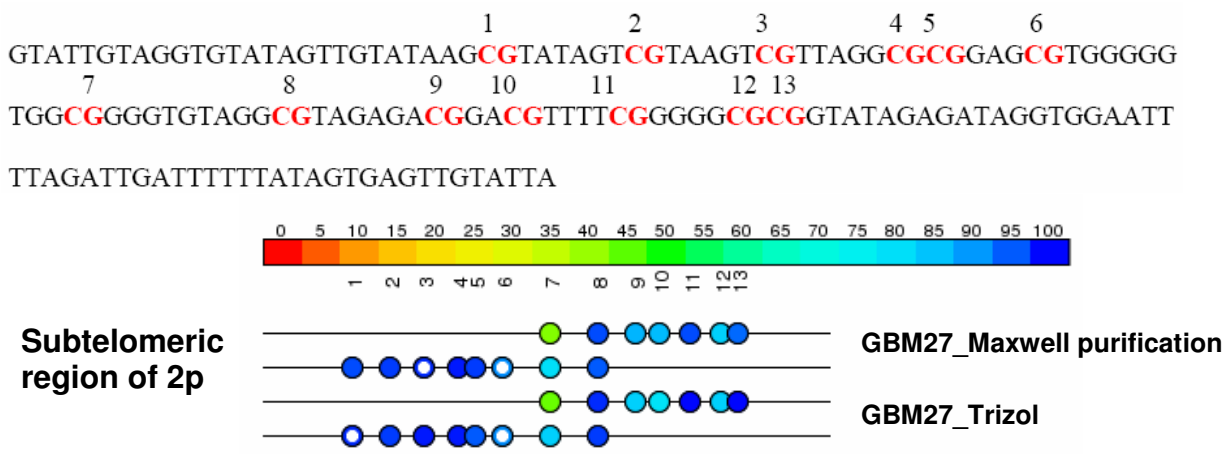
T_for … DNA isolated from Trizol and sequenced with primer MP_HC18 FWD (table 4.4)

T_rev … DNA isolated from Trizol and sequenced with primer MP_HC18 REV (table 4.4)

C_for … DNA isolated from Chaps and sequenced with primer MP_HC18 FWD (table 4.4)

C_rev … DNA isolated from Chaps and sequenced with primer MP_HC18 REV (table 4.4)

a)



b)

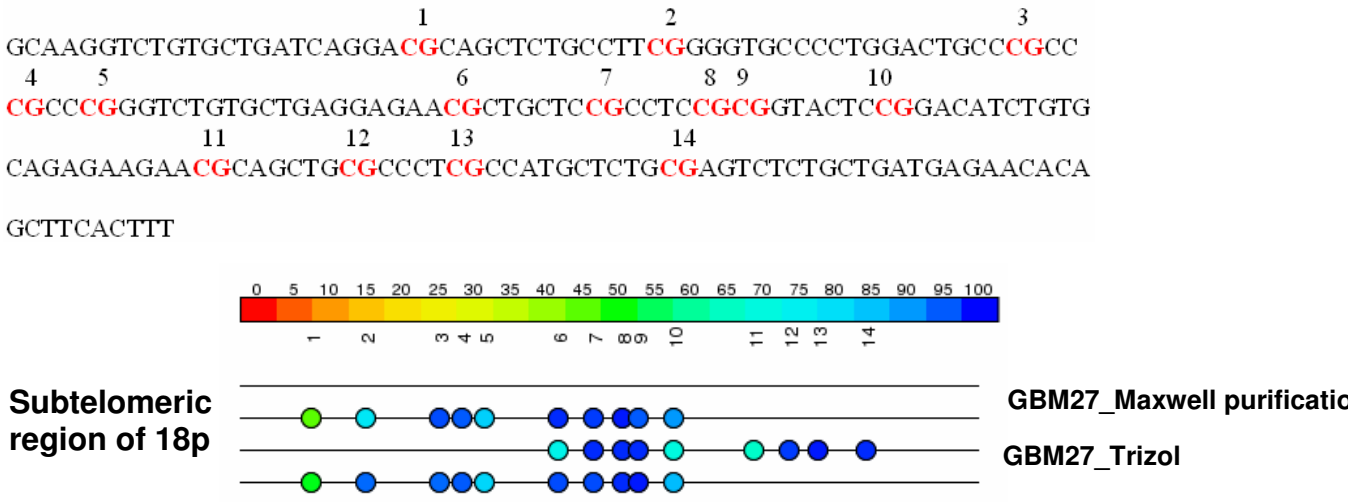


Figure 5.24 **Methylation of CpG islands from the subtelomeric region of chromosome 2p and 18p.**
DNA isolated with the Trizol and Maxwell method were analysed from tumour tissue GBM27.
Heatmap indicate color code for % of methylated C's at the respective CpG-position. Circles that are not filled indicate that there is a peak from another base in the sequencing chromatogram higher than the peak for the CpG and thus the quantity can not be determined accurate. Primers taken for amplification are located proximate before the first and after the last CpG-positions.
(a) Subtelomeric region of chromosome 2p includes 13 CpG positions and
(b) Subtelomeric region of chromosome 18p includes 14 GpG positions. Lines represent the sequencing reactions performed from 5' and 3' site of the amplicon. This information from both sequencings should be identical, but was sometimes missing (in case of Maxwell-DNA) or partially available due to problems with the sequencing reaction as described in the text.

Next, the subtelomeric chromosome 2p epigenetic state of 5 grade II and III (DA + AA) and 7 grade IV astrocytomas was determined.

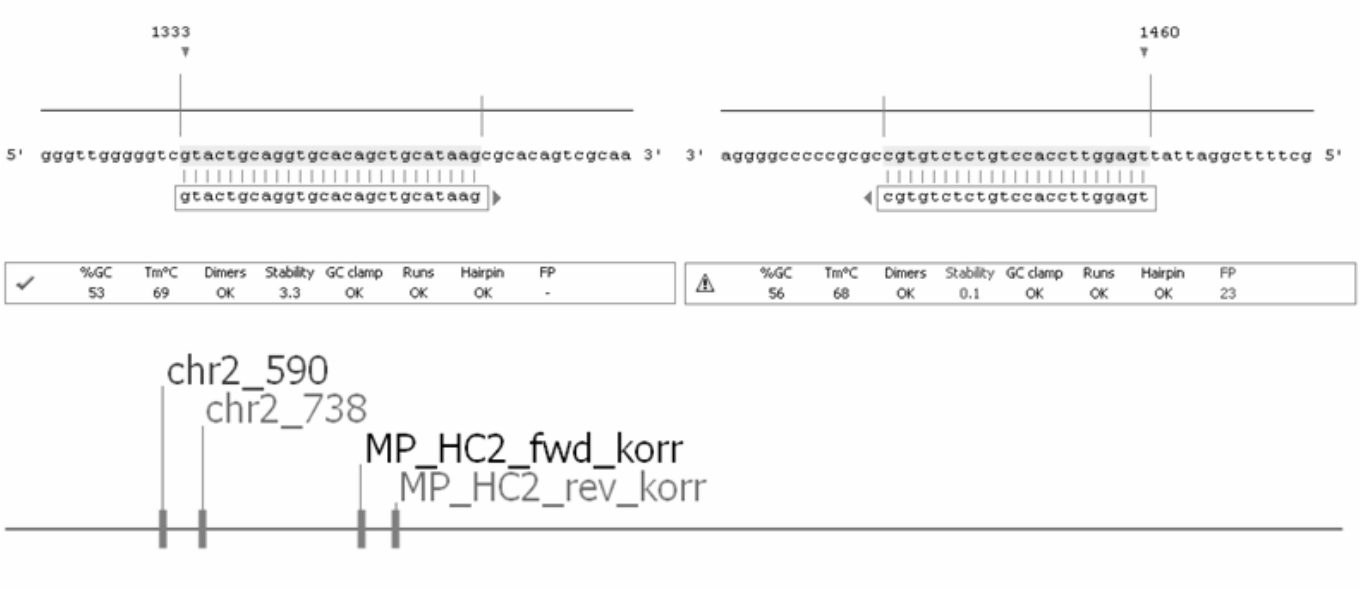


Figure 5.25a **Linking of primers MP_HC2_FWD and MP_HC2_REV to the genomic sequence of subtelomeric region of chromosome 2p** in CloneManager 9 program. The primer sequence in this figure is the not converted sequence, because they are alingned against the non-converted genomic sequence of the subtelomeric region of chromosome 2p.
Line in the lower panel represents sequence of chromosome 2p end ….. Primers for 2pTERRA expression and CpG island-specific PCR are shown).

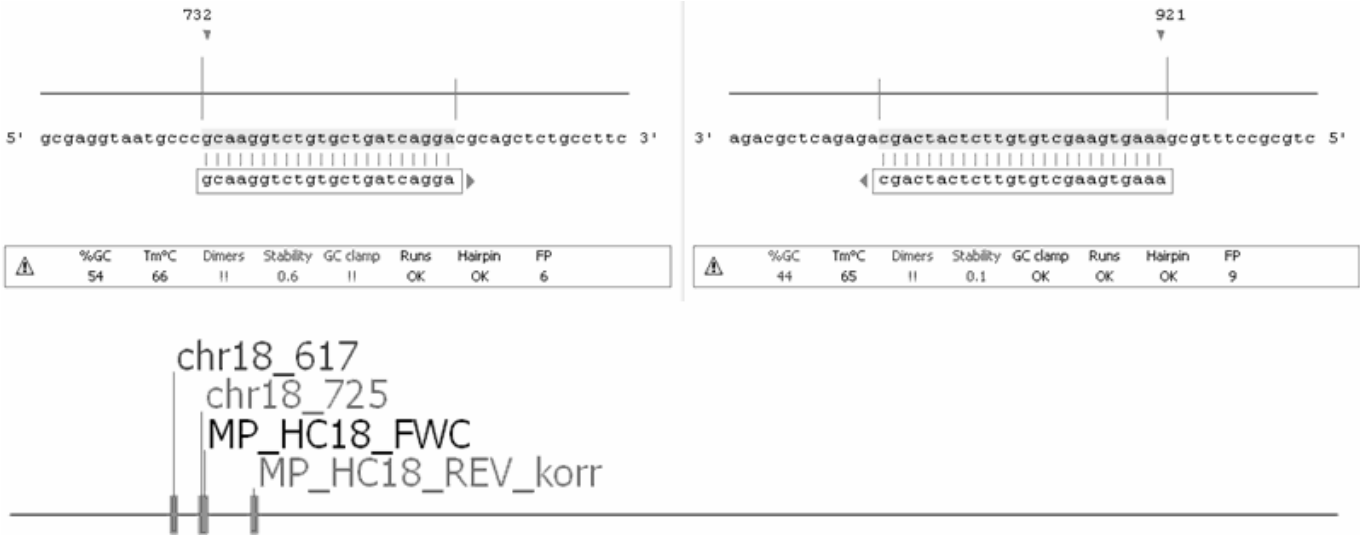


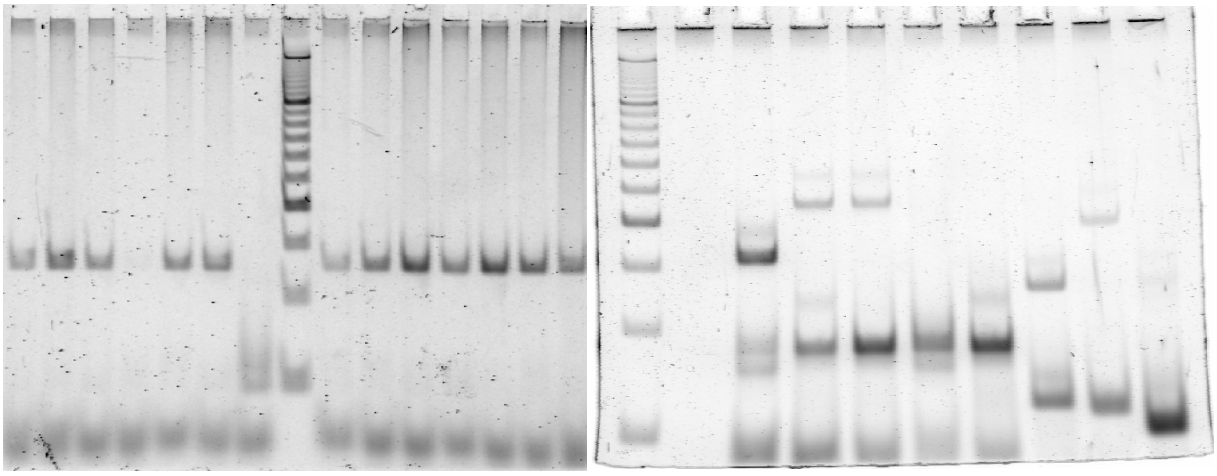
Figure 5.25b **Linking of primers MP_HC18_FWD and MP_HC18_REV to the genomic sequence of subtelomeric region of chromosome 2p** in CloneManager 9 program (primers for 18pTERRA expression and methylation-specific PCR are shown in the lower panel).
The primer sequence in the upper panel of the figure is the not converted sequence, because they are alingned against the non-converted genomic sequence of the subtelomeric region of chromosome 18p.

Specific PCR was first done with primers as described in literature [24] and with converted primers (material section, Table 4.4) as those shown for the chromosomal end 2p and 18p in

Figure 5.25a and 5.25b, respectively. The positions of the primers for TERRA PCR expression assays analyses are located close to the CpG islands as indicated. Lots of the CpG-positions could not be determined in the sequencing reaction when using the same primers as for PCR (Figure 5.24). The quantitative sequencing method of Qiagen needs few base positions (15 to 30) before correct sequence information can be obtained. New reverse primers extendet at the 5' end with a spacer sequence and a T7 primer sequence have been constructed (see Methods Table 4.4) Converted DNA was amplified with the new and longer primers, and by analysing the amplicons by PAGE gel, it can be seen that they produde more primer dimers than the original primers (Figure 5.26).

To circumvent this problem, amplicons with the new and longer reverse primers were purified on a 3% agarose gel before sequencing (Figure 5.26b). Pieces of the agarose gel containing respective amplicons were cutted out from gel and sent directly to Qiagen for DNA isolation from the gel and subsequent bisulfite sequencing.

a) b)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1 2 3 4 5 6 7 8 9 10

Figure 5.26(a+b) **PCR of bisulfite converted DNA**

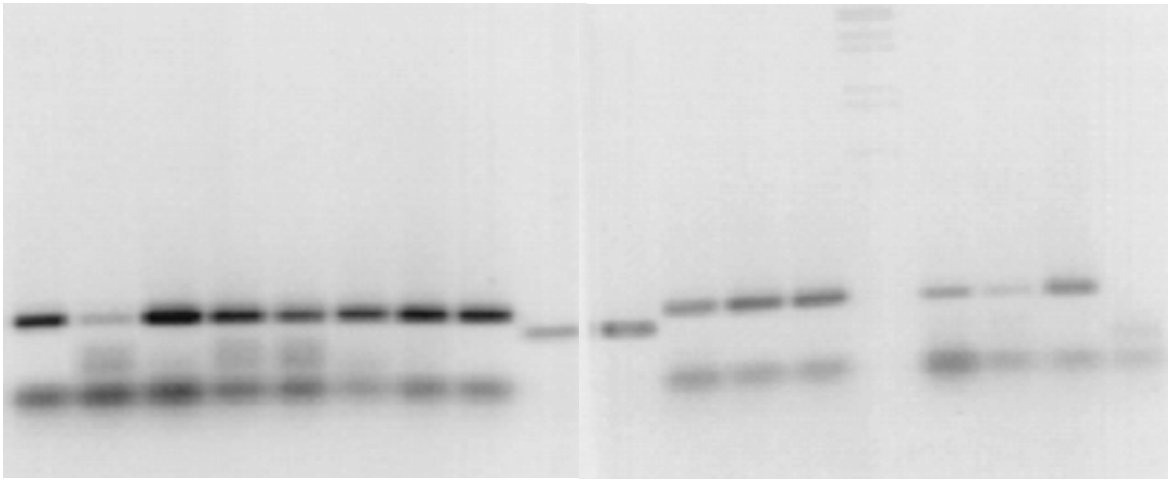
(a) PAGE-Gel with amplicons for primers MP_HC2 FWD and MP_HC2 REV (as described in materials and methods). Length of PCR product is 129 bp. GBM 2 (lane 1), DA 10 (lane 2), GBM 21 (lane 3), DA 11 (lane 4), GBM 28 (lane 5+6), GBM 25 (lane 7), NTC (lane 8), 50bp marker (lane 9), GBM 10 (lane 10), GBM 5 (lane 11), AA 2 (lane 12), AA 3 (lane 13), DA 5 (lane 14), DA 6 (lane 15);

(b) 50bp Marker (lane 1), chr2 with new primer on Trizol DNA (lane2), chr2 with new primer on Maxwell DNA (lane 3), chr18 with new primers on Trizol DNA (lane 4), chr18 with new primers on Quiagen DNA (lane 5), non template control for new primers chr2 (lane6), non template control for new primers chr18 (lane7), chr2 with original primers on Quiagen DNA (lane8), chr18 with original primers on Trizol DNA (lane9), chr2 with original primers on Trizol DNA (lane10)

New Primers ... pimers (MP_HC2 FWD + MP_HC2_REV_S) and (MP_HC18 FWD + MP_HC18_REV_S) for chromosome 2p and chromosome 18p, respectivively

Chr2 and chr18 … chromosome2p and chromosome18p, respectively

a) b)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 5.27(a+b) **PCR of bisulfite converted DNA with primers specific for subtelomeric region of chromosome 2p.**

(b) 3% agarose gels to purify amplicons produced with pimers MP_HC2_FWD and MP_HC2 REV_S (as described in Materials and Methods). Length of amplicon is 156 bp. As a control, product with primers MP_HC2 FWD and MP_HC2 REV is also loaded on the gel. DA 10 (lane 1), GBM 2 (lane 2), AA 3 (lane 3), DA 6 (lane 4), GBM 21 (lane 5), DA 11 (lane 6), GBM 25 (lane 7), GBM 28 (lane 8), control with primers MP_HC2 FWD and MP_HC2 REV (lane 9), GBM 10 (lane 10), GBM 5 (lane 11), DA 5 (lane 12), λ-Marker (lane 13), KM-HY (lane 14), T98G (lane 15), Saos-2 (lane 16), NTC (lane 17), control with primers MP_HC2 FWD and MP_HC2 REV (lane 18);

Ratios of methylated versus unmethylated were obtained for 12 out of 13 CpG positions in all astrocytomas analysed.

Sequencing results (Table 5.13a+b and shown in Figure 5.28) revealed, that CpG islands in subtelomeric region on chromosome 2p are highly methylated and there is little inter-individual variation across all tested astrocytomas (Average ± SD = 87 ± 5 %) with some light methylated (<65%) CpGs.

Unmethylated CpGs were detected in SAOS-2, an ALT tumour-derived cell line, in contrast to dense methylation observed in T98G, a telomerase-positive tumour-derived cell line.

Analysed epigenetic state did not differ between tissue sample groups of low and high tumour grades (P=0.6506) but between groups of low and high 2p TERRA levels (P=0.0127).

low 2p TERRA	RQ 2pTERRA	RQ TL	1	2	3	4	5	6	7	8	9	10	11	12	13	mean+SD	median
DA5	0.2369	0.36	89	77	85	93	90	95	93	92	97	90	n.a.	80	96	89.8	91
DA11	0.7585	8.15	86	***65***	***65***	78	73	86	82	92	92	78	n.a.	75	94	80.5	80
AA3	0.0894	8.90	84	75	83	92	86	83	76	91	86	***56***	n.a.	79	95	82.2	83.5
Gbm 2	0.0259	4.75	93	83	73	95	76	92	87	93	95	88	n.a.	72	98	87.1	90
Gbm21	0.0331	8.97	88	77	85	92	90	93	75	92	80	78	n.a.	83	94	85.6	86.5
Gbm27	0.3346	1.33	96	97	98	99	97	91	80	95	83	79	100	83	100	83.9	83
Gbm28	0.1801	5.25	82	76	90	93	90	96	94	94	97	93	n.a.	82	98	90.4	93

Table 5.13a Methylation of CpG islands in the subtelomeric region of chromosome 2p in samples with high 2p TERRA expression. Methylation level is shown for the individual CpG islands and also mean and median of methylation of all CpG islands is listed in the table.
Italic, bold … light methylated CpGp positions

high2p TERRA	RQ 2pTERRA	RQ TL	1	2	3	4	5	6	7	8	9	10	11	12	13	mean+SD	median
DA6	0.8444	82.34	91	76	84	81	***63***	***47***	95	87	97	96	n.a.	91	96	83.7	89
DA10	0.7170	7.74	85	77	87	93	87	93	71	94	76	***52***	n.a.	77	94	82.2	86
AA2	0.3075	16.21	96	93	86	96	86	94	96	99	98	100	96	92	99	94.7	96
Gbm 5	1.1464	8.17	81	78	71	88	84	88	95	93	97	93	n.a.	92	89	87.4	88.5
Gbm10	1.2572	26.96	85	***63***	79	92	87	93	*58*	86	91	85	n.a.	86	95	83.3	86
Gbm25	0.3415	6.59	89	79	90	94	91	96	93	95	98	95	n.a.	88	97	92.1	93.5
T98G	0.0218	10.96	99	90	95	93	95	97	99	95	99	n.a.	n.a.	n.a.	n.a.	95.8	95
SAOS2	0.1134	37.46	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	32	28	13	2	3	14	29	17.3	14

Table 5.13b Methylation of CpG islands in the subtelomeric region of chromosome 2p in samples with high and low 2p TERRA expression. Methylation level is shown for the individual CpG islands and also mean and median of methylation of all CpG islands is listed in the table.
Italic, bold … light methylated CpGp positions

(a)

```
                                        1           2          3          4 5        6
GTATTGTAGGTGTATAGTTGTATAAGCGTATAGTCGTAAGTCGTTAGGCGCGGAGCGTGGGGG
      7                 8           9    10        11         12 13
TGGCGGGGTGTAGGCGTAGAGACGGACGTTTTCGGGGGCGCGGTATAGAGATAGGTGGAATT

TTAGATTGATTTTTTATAGTGAGTTGTATTA
```

(b)

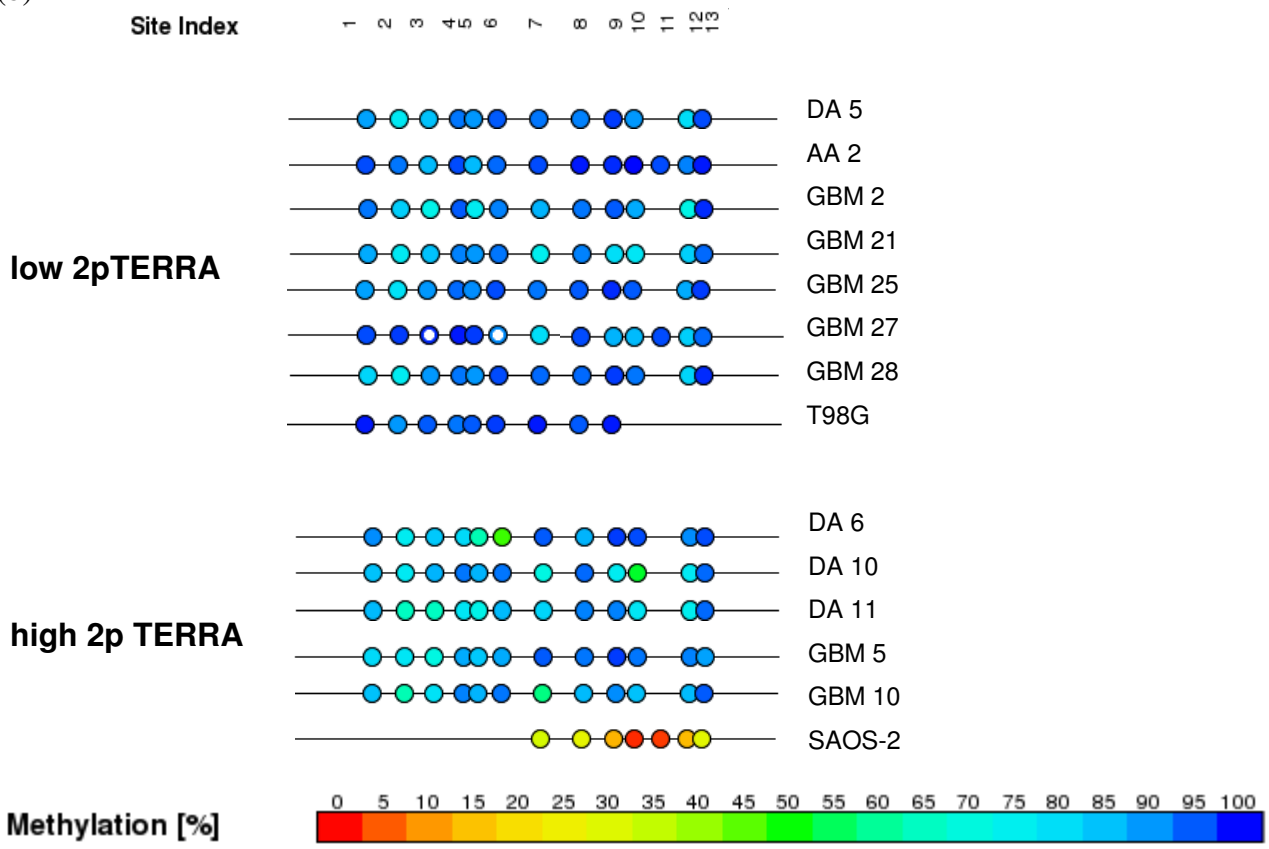


Figure 5.28 (a+b) **Methylation of CpG islands in the subtelomeric region of chromosome 2p.**
(a) Sequence of subtelomeric region of chromosome 2p. 13 CpG islands are included in this region. CpG islands are labelled in red and by a serial numbers from 1 to 13.
(b) Methylation of CpG islands in astrocytoma tissue samples. Samples are splitted into high 2p TERRA expressing samples and low 2p TERRA expressing samples. Colours of circles show percentage of methylation of individual CpG islands as indicted by the heat map.

Mean methylation of the CpG-island for 2p TERRA was compared between tumour samples expressing 2p TERRA at a low and high level. A significant difference could be demonstrated with the mean methylation status of all CpG-positions analysed at the CpG-island (Figure 5.29). High 2p TERRA expressing samples are less methylated, than samples with low expression of 2p TERRA.

In contrast, there was no significant difference when mean methylation was compared between grade II/III and grade IV astrocytic tumour samples (Figure 5.30).

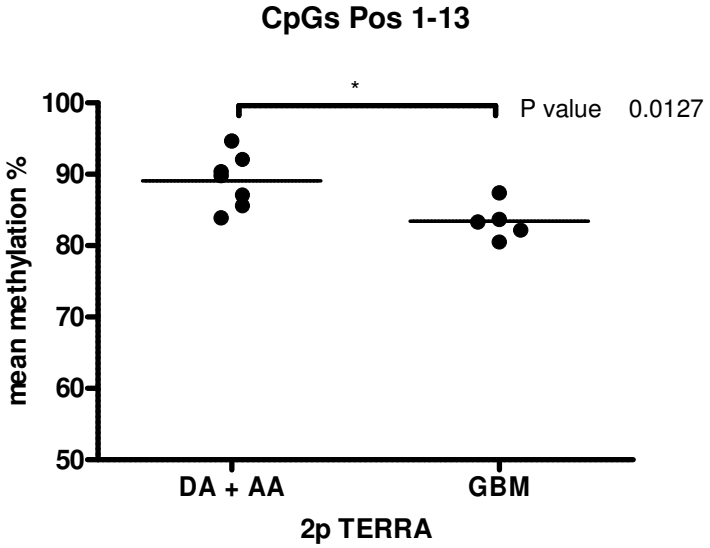


Figure 5.29 **Differences in mean methylation between high and low 2p TERRA expressing tumour samples.** There is a significant (*) difference in the mean methylation of all 13 CpG islands and the expression level of 2p TERRA when testing data with unpaired t-test with Welch´s correction.

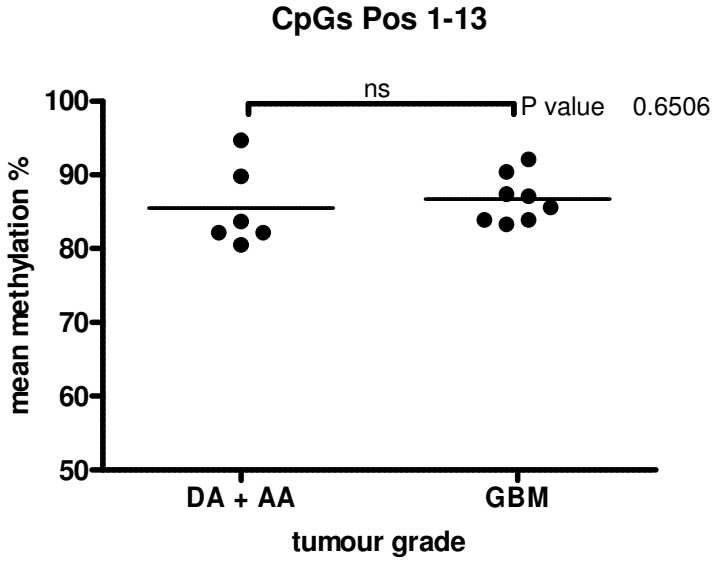


Figure 5.30 **Difference in mean methylation of all 13 CpG islands in grade II/III and grade IV tumour samples.** The unpaired t-test with Welch´s correction showed no significant difference in the mean methylation between low grade tumour samples and high grade tumour samples. The methylation result of the single grade II probe is marked by an open circle.

Comparing the telomere maintenance mechanism (TMM) used with the methylation of 2p-subtelomeric region; no correlation was found, neither with telomerase activity nor with telomere length. No significant difference in mean methylation was detected when tumours with negative and positive telomerase activity or long and short telomeres were tested (Figure 5.31).

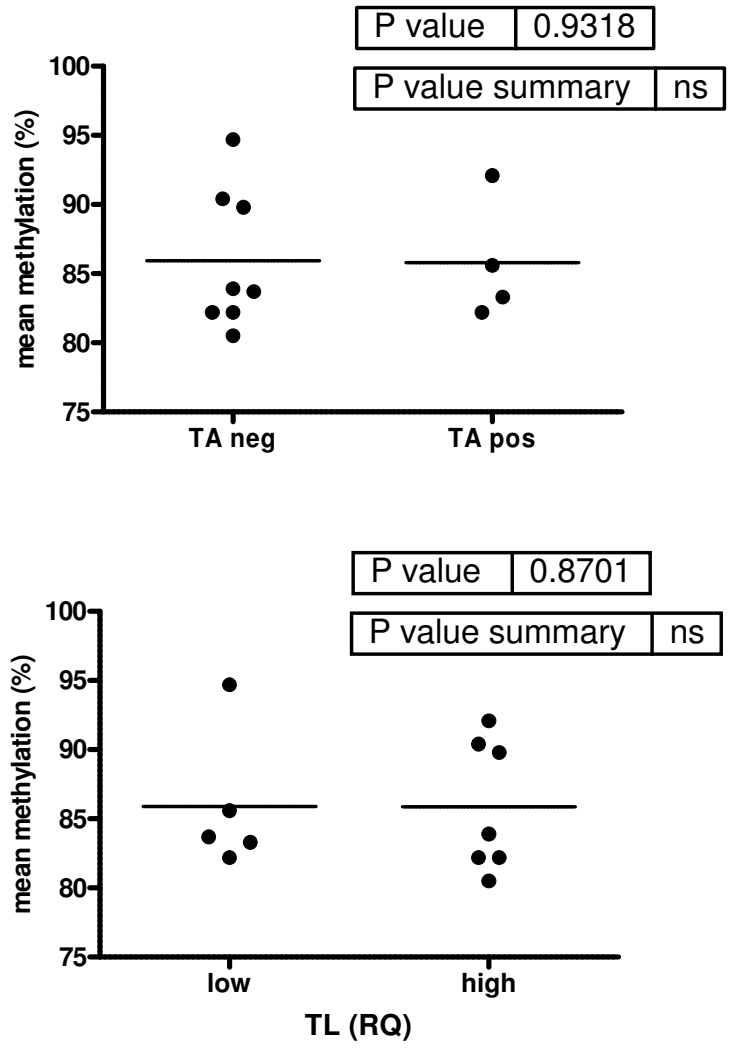
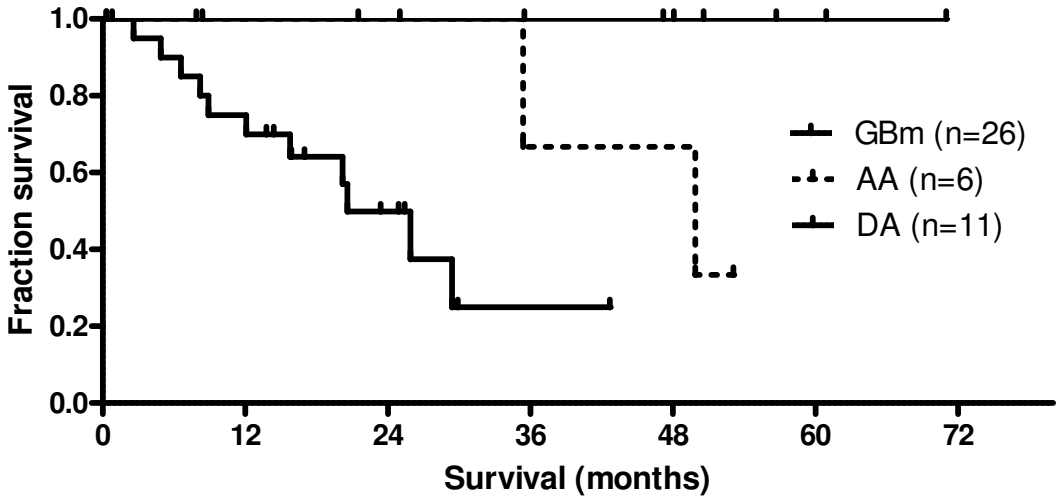


Figure 5.31 **Comparison with unpaired T-test with Welch´s correction of telomerase activity and telomere length status with methylation of subtelomeric region of chromosome 2p**

Median of RQs of telomere length was used for grouping tumours in long and short telomeres.

5.5 Comparison with clinical data

For 27 out of 28 (96%) and for 17 out of 18 (94%) patients with high (GBM) and low (AA, DA) grade clinical data were available, respectively.
For correlating survival of patients with diagnosed tumour grade, TL, overall TERRA, 2p TERRA and 18p TERRA, the median over all tumour samples was used to classify patients in groups with low and high values. The two resulting survival curves were compared to differ significantly by the log-rank (Mantel-Cox) test.
Clinical data demonstrate that 2 (Figure 5.32).

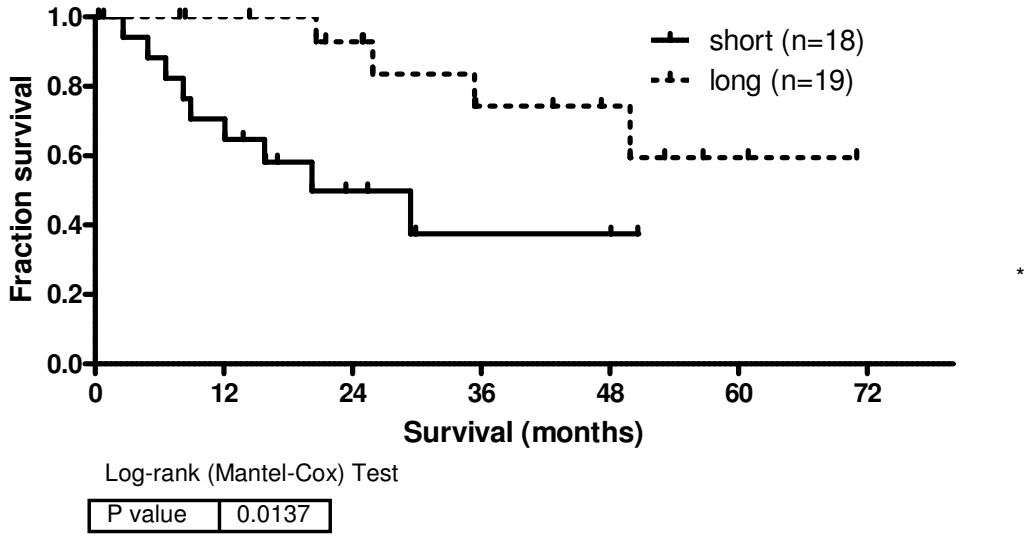


Logrank test for trend

P value	0.0009
P value summary	***

Figure 5.32 **Survival of patients compared with tumour gradeII/III und IV!).**
Mean survival GBM … 20.6 months
Mean survival AA … 49.9 months
Mean survival DA … > 72 months

Next, survival of patients suffering from low and high grade astrocytomas was compared with telomere length. This result shows that patients with longer telomeres survive significantly longer (mean survival= >72 months) when compared to patients with short telomeres (mean survival=20.2 months) shown in Figure 5.33.



Log-rank (Mantel-Cox) Test

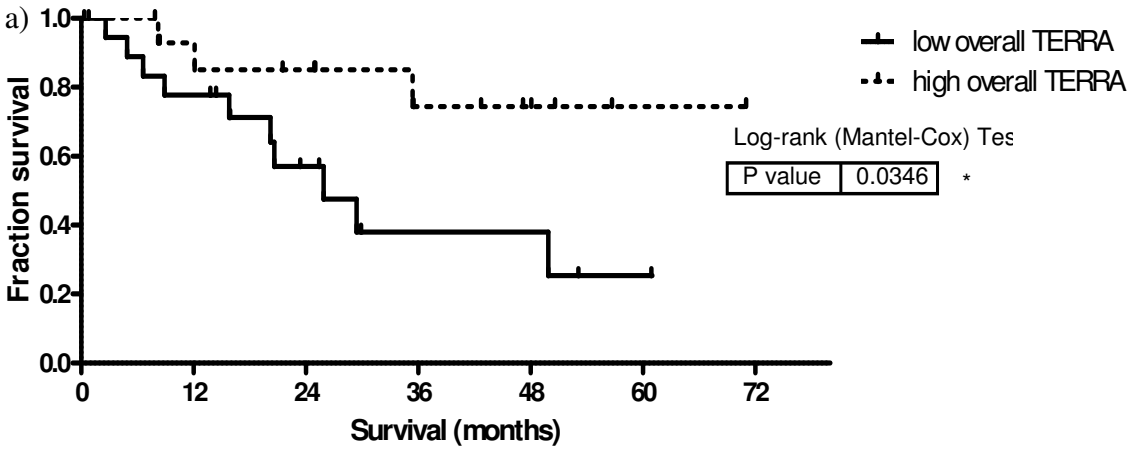
P value	0.0137

Figure 5.33 **Correlation of telomere length with survival.** Higher telomere lengths show a significantly better outcome for patients suffering from astrocytomas. The median of the RQs of telomeres was taken to classify long and short telomeres.

Mean survival for long telomeres … >72 months

Mean survival for short telomeres … 20.2 months

The correlation between survival of patients and TERRA expression (overall TERRA, 2p TERRA and 18p TERRA) and methylation gave following results (Figure 5.34). High expression of overall TERRA correlated significantly with a longer survival (mean survival high overall TERRA expression>72months and for low overall TERRA expression = 25.9 months), while 2p TERRA correlated with longer survival only, when the median of low grades was used for the classification of patients in groups with low and high 2p TERRA expression (mean survival high 2pTERRA expression>72months and for low 2pTERRA expression = 29.4 months). 18p TERRA never showed a correlation with survival, leading to the conclusion, that not all but some specific TERRA transcripts might be responsible for prediction of a longer patient survival.



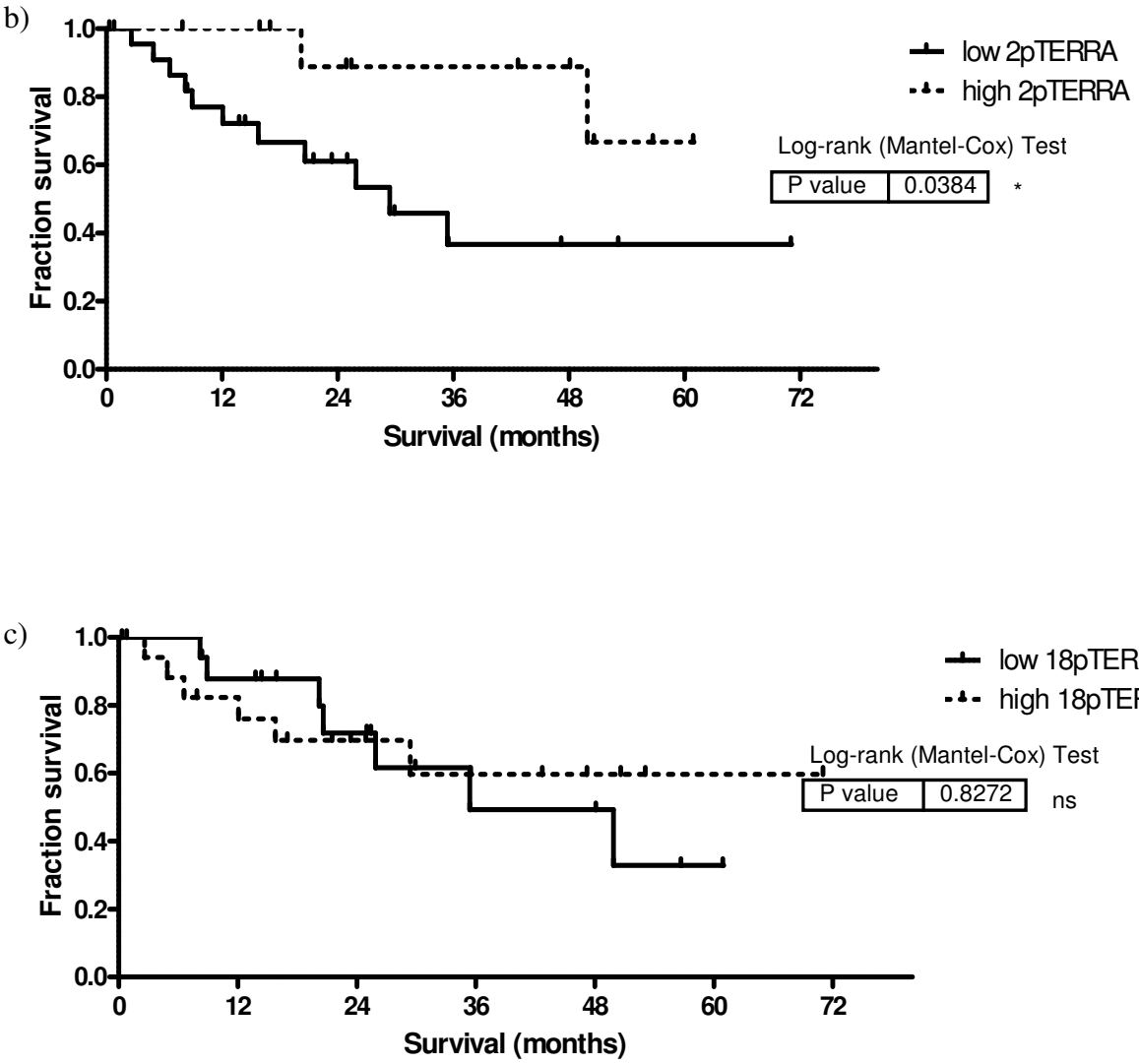


Figure 5.34 (a–c) Kaplan-Meier survival curves for correlation of overall, 2p and 18p TERRA expression with survival

(a) High overall TERRA expression correlates significantly with longer survival,

Mean survival for high overall TERRA expression … >72 months

Mean survival for low overall TERRA expression … 25.9 months

(b) high 2p TERRA expression correlates with longer survival,

Mean survival for high 2pTERRA expression … >72 months

Mean survival for low 2pTERRA expression … 29.4 months

(c) 18p TERRA expression shows no correlation with survival

6 Discussions

Linear chromosomes of eukaryotic cells are capped by telomeres, which protect the ends of chromosomes from being recognized by DNA repair mechanism as ds DNA breaks and from signalling DNA damage. Telomeres achieve this, by interaction of telomeric DNA and telomere associated proteins. In normal human cells, telomeres consist of 4 to 12 kb of ds DNA of 5´-TTAGGG-3´ repeats [41], terminating in an ss DNA 3´-overhang of the G-rich sequence [42].

In this study I have determined the telomere length and the RNA expression of telomeres (TERRA molecules) in tumour samples of patients suffering from astrocytic brain tumours and in non-tumour tissue obtained from epileptic foci. Beside this in vivo material I analyzed in vitro cell lines established from astrocytic brain tumours and from cell line Saos2, derived from a primary osteosarcoma. Saos2 is described in the literature as telomerase negative but ALT positive cell line [43]. The astrocytoma cell lines analysed all utilize telomerase activity as TMM and not ALT (D.Mejri personal communication, data not shown). For measurement of telomere length I isolated DNA by two different methods, namely Trizol and Maxwell DNA Purification Kit. The amounts I isolated by Trizol method were less, compared to DNA isolated with Maxwell DNA Purification Kit. The purity of the isolated DNA by both methods was generally in the range of 1.75 – 2.0 (ratio of absorbance at 260 nm to absorbance at 280 nm). This indicates a low content of protein which could interfere with the following analyses. In a first set of experiments, I used Trizol isolated DNA for successful determination of telomere length by Real time qPCR. I found that DNA isolated by this method is not suitable for TRF analysis, because it was not or only partially digestible by restriction enzymes. Kong et al [44] described DNA isolated from rat tissue by Trizol method as a high-quality DNA with an average amount of 150 -300 ng/µl and a purity of 1.75 - 1.95. I did not found any published data for astrocytomas after extensive search in the literature. In a second set of experiments, I used for TRF analysis DNA isolated by the Maxwell DNA Purification Kit. The DNA was directly isolated from tissue samples or cell pellets, or alternatively from cells after mild protein extraction with Chaps detergence from the remainders as described by Henson et al [30]. This DNA from high quality and purity was completely digestible and therefore suitable for telomere length determination by TRF analyses. Next, I compared the two basically different methods PCR and TRF analyse for measuring the telomere length in vivo and in vitro. I found a weak but significant correlation between the two methods (P=0.427; p=0.0212). This allowed me to use the more simple

method PCR to determine the absolute telomere length of tumour samples, similar as measured on blood cells published by O´Callaghan [32]. Furthermore, all DNA from tissue samples and cell lines isolated by the Trizol method was adaptable. Relative T/S ratios of the PCR results were converted to absolute mean TRF telomere lengths in kb using linear interpolation.

My results of telomere length measurement demonstrate a mean telomere length over 17 kb for diffuse astrocytoma (DA) and anaplastic astrocytoma (AA), but not in glioblastoma multiforme (GBMs). This indicates a higher content of ALT positive tumours in low grade tumours as compared to GBM.

Hakin et al [39] defined a telomere length of over 17 kb as a key feature of ALT. Le et al [45] detected telomerase activity in 33% of DA, 45% of AA and 89% of GBM, indicating a higher percentage of low grade tumours using the ALT mechanism compared to GBMs. Thus my measured telomere length in astrocytomas correlates with data from literature [39, 45].

I quantified the expression of overall TERRA and chromosome specific TERRA for chromosome 2p and 18p. I found the expression of overall TERRA significantly decreased in GBM compared to AA and DA. Astrocytoma cell lines contained TERRA levels found as in GBM, the ALT cell line as in DA. Consistent with these results, Schoeftner and Blasco [20] found TERRA significantly downregulated in advanced stages of different human types of cancer compared to normal tissue. This suggests that down regulation of TERRA is a general mechanism by disease progression and is associated with undifferentiated cell stages.

The expression of specific chromosome 2p and chromosome 18p TERRA was measured by Real Time PCR with self-designed primers within the sub-telomeric region. In contrast to overall TERRA, the expression found for 2p and 18p TERRA was not significantly altered and depending on tumour grade. I could not find any results in the literature for quantification of chromosome specific TERRA expression. Why chromosome specific TERRA levels do not reflect overall TERRA remains to be further investigated.

For the subtelomeric region of chromosome 2p I determined the methylation state of CpGs as described [24]. When comparing tumours showing a high 2p TERRA expression with tumours of low TERRA level the average methylation was significantly decreased. Thus methylation correlates inversely with TERRA expression. Like 2p TERRA expression methylation state also did not show a significant change with grade of the tumour.

Methylation in CpG dinucleotides is a well known marker for transcriptional silencing, and sequences maintained as heterchromation are usually heavily methylated [46].

By analyzing my data I found a strong positive correlation between the telomere length and the overall TERRA expression. This finding is supported by Ng et al [24]. They report a reduced TERRA expression in telomerase-positive cells compared to normal and ALT cells. In contrast to cells with telomerase activity, ALT cells like the SAOS-2 cell line I analysed, have longer telomeres and a higher content of TERRA. TERRA oligos were shown to inhibit telomerase activity [28]. Thus, TERRA silencing could maybe important in order to facilitate telomerase activity at telomeres.

I compared my result with patient data like the diagnosis (grade of the tumour) and the survival of patients. Patients with the diagnoses DA and AA survived significantly longer compared to patients with GBMs (logrank test for trend p=0.0009). Patients suffering from GBM have mean survival of 20.6 months, AA 49.9 months and DA > 72 months Fitting published data [2] with a mean survival GBM patients of <12 months, AA 24 - 60 months and DA 44 - 96 months.

Long telomeres correlate significant with a longer survival of patients (p=0.0137). Patients with long telomeres showed a mean survival of over 72 month, compared to a mean survival of 20.2 month for patients with short telomeres. Also a high expression of overall TERRA correlates significantly with a better outcome (p=0.0346). Mean survival for high and low overall TERRA expression is > 72 months and 25.9 months, respectively. Both, high telomere length and high expression of TERRA are both features of ALT [24].

This findings are supported by published data [30], that ALT positive tumours have a better outcome than telomerase positive tumours.

In addition to overall TERRA also low 2p TERRA expression correlates with a poor outcome (p=0.0384). Mean survival for patients with high and low 2pTERRA expression are >72 months and 29.4 months, respectively. 18p TERRA did not show to correlate with the prognosis (p=0.8272). After extensive search I found no published data for the influence of chromosome specific TERRA on the survival of patients.

Maybe TERRA could be a promising candidate a prognostic and potentially prognostic marker in astrocytomas. To fully understand mechanisms and functions of TERRA, futher studies would be needfull.

Curriculum vitae

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