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TELOMERE REPEAT FRAGMENT SIZES DO NOT LIMIT THE GROWTH POTENTIAL OF UTERINE LEIOMYOMAS

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SUMMARY: We have compared the length of telomere repeat fragments (TRF´s) in 19 uterine leiomyomas from 6 patients with the corresponding myometrium. The advantage of this study of TRF length is that cells from uterine leiymyoma and cells from corresponding myometrium do not contain any considerable proportions of other cells as revealed by analysis of clonality.
In all tumor samples a loss of TRF length ranging from 1120 to 4690 bp was noted. There was no correlation between tumor volume or size of tumor population as revealed by histological examination and loss of TRF length. From the obtained TRF length data (an average myometrial TFR length of 13 kb and an average loss of TRF length in myoma cells of 83 bp per cell division) we concluded that TRF length reduction does not limit the growth potential of uterine leiomyomas.

Due to the so called end-replication problem [1, 2], there is a loss of DNA at the end of chromosomes with every cell cycle [3-6]. To avoid a loss of coding sequences and to protect the end of chromosomes from chromosome fusions [7] the telomeric part of human chromosomes consists of the repetitive sequence [TTAGGG]n [8]. It is believed that the telomeric repeat forms specialized structures either by association with telomere-specific proteins [9] or by non-Watson-Crick base pairing of the G-rich-single-stranded-overhang of the telomeric repeat [10, 11]. Harley et al. [12] have assumed that there is a relationship between the telomeres, aging, and tumorigenesis in that shortening of TRF length under a critical size causes cell cycle exit. Tumor cells thus have to express telomerase activity to gain unlimited growth [5, 12, 13].
While the telomere hypothesis is primarily based on the results of in vitro investigations, little is known about the dynamics of the telomeres in vivo. Some authors have reported a

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decreased mean telomeric repeat fragment (TRF) length in some primary tumors compared to a control tissue, but in the same studies also increased and constant lengths of TRFs are described for other tumor samples [14-23]. Although it has frequently been suggested that constant TRF lengths correspond to an expression of telomerase activity it should be kept in mind that in most of these in vivo TRF investigations, however, the control-tissue was not the tissue the tumor has originated from. Some of the in vivo investigations are weakened by the fact that as controls even completely different tissues or tissues from other donors have been used e.g. blood in investigations of solid tumors. In addition, some of the tumors investigated contain rich parts of stromal tissue not belonging to the tumor.

Due to these reasons we investigated the TRF dynamics in a tumor where both tumor and appropriate control tissue are easily available. For our TRF investigation we used uterine leiomyomas, the most frequent tumors in women in their reproduction phase [24]. Uterine leiomyomas are benign mesenchymal tumors originating from the smooth muscle of the myometrium [25]. Both tumor and myometrial tissue do not contain considerable proportions of other cells. Additionally, several leiomyomas of different sizes are frequently found within one uterus offering a possibility to correlate various tumors from one patient to the TRF size of the corresponding myometrial tissue.

Using this system (uterine leiomyoma versus myometrial tissue) as the source for the present TRF study we have also checked whether or not the lack of telomerase activity necessarily limits the growth potential of human tumors.

MATERIAL AND METHODS

For our investigations we used samples of 19 uterine leiomyomas from 6 patients. In every case the corresponding myometrial tissue was also investigated.

Directly after surgery the volume of the tumor was determined. Part of each tumor was frozen in liquid nitrogen and stored at -80°C for the molecular investigations. Another part was stored in Hank´s solution for cytogenetics and the remaining part was fixed in 5 %(v/v) formaldehyde for histological examinations.

Cytogenetic Studies. For short time cell cultures and cytogenetic analysis, routine methods described elsewhere [26] have been applied.

Histological Examinations. Using standard methods, 10 μm HE stained sections were prepared from the uterine leiomyomas after paraffin embedding. Two areas of each tumor section were used for determination of its cellularity. Based on the cellularity of the tumors and their volume we were able to determine roughly the total size of the tumor cell population.

DNA Isolation. The tumor samples were minced first using a scalpel and then in a mortar under liquid nitrogen. Following steps were performed as recently described [27].

Clonality Assay. The clonality of the tumor samples was determined according to a modification of the method described by Noguchi et al. [28]. Only one PCR using the primer pair 2A and 2B was run containing 10 mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 100 μM ATP, 100 μM TTP, 100 μM GTP, 100 μM CTP, 1 μM primer 2A, 1 μM primer 2B, 10 ng/μl Hpa II digested DNA, and 1 unit/100 μl Taq polymerase for 40 cycles (1 min 94°C, 2 min 58°C, 3 min 72°C). PCR products were

digested with BstX I (Promega/Serva, Heidelberg, Germany) at 55°C for 2 hours and separated on 2 % agarose gels (50V, 7 cm).

Southern Hybridization. Genomic DNA was digested with Alu I and Hinf I (Promega/Serva, Heidelberg, Germany) overnight at 37°C. 5 μg DNA per lane were separated on 0.8 % agarose gels (50V, 14 cm). Prior to blotting, the gels were depurinated twice in 0.25 M HCl for 15 minutes, denatured twice in 0.5 M NaOH, 1.5 M NaCl for 15 minutes, and neutralized three times in 3 M NaCl, 0.5 M Tris, pH 7.5, for 10 minutes. DNA was vacu-blotted on nylon membranes in 20 x SSC (pH 7.0) and fixed by baking. For hybridization a telomere-specific probe $[TTAGGG]_n$ was generated in a PCR reaction containing 10 mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 100 μM ATP, 66 μM TTP, 34 μM digoxigenin-UTP, 100 μM GTP, 100 μM CTP, 1 μM primer $[GGGTTA]_3$, 1 μM primer $[TAACCC]_3$, 10 ng/μl DNA of a fibroblast cell line, and 1 unit/100 μl Taq polymerase by amplification for 30 cycles (2 min 94°C, 2 min 57°C, 3 min 72°C). Hybridization was performed with 0.1 % (v/v) of PCR mix containing the digoxigenin labeled $[TTAGGG]_n$ probe in 50 % formamid, 5 x SSC, 2 % blocking reagent (Boehringer, Mannheim, Germany), 0.1 % N-lauroylsarcosine, and 0.02 % SDS overnight at 42°C, followed by high stringent washes twice in 2 x SSC supplemented with 0.1 % SDS at room temperature for 5 minutes and twice in 0.1 x SSC supplemented with 0.1 % SDS at 68°C for 15 minutes. The digoxigenin-labeled probe was detected by the dig luminescent detection kit (Boehringer, Mannheim, Germany) and documented on X-ray-films. Each lane was scanned with a densitometer (Desaga, Heidelberg, Germany). For calculation of the average TRF length, the upper and lower border of a TRF length distribution were defined at the half of maximal optical density (fig. 1).

RESULTS

Cytogenetics. All tumors used in this study had an apparently normal karyotype without clonal aberrations.

Clonality Assay. One out of 6 patients was heterozygous for the X-linked PGK gene. The clonality assay showed monoclonal origin for all 5 tumors from this patient as revealed by

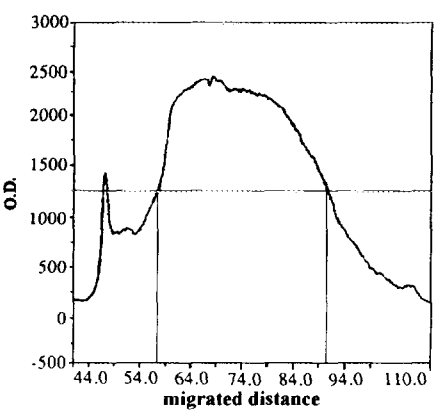

Figure 1. Densitometrical TRF length profile to the corresponding tumor sample 183.1 shown in fig. 4.

the loss of one of the two restriction fragments of the corresponding myometrial tissue (fig. 2). The clonality assay applied herein also allows for a clear demonstration of purity of the cells used. A considerable contamination with cells not belonging to the tumor can thus be ruled out.

Histological Examinations/Size of Tumor Cell Population. The volumes of the uterine leiomyomas included in this study varied from 0.5 cm^3 to 214 cm^3 (tab. 1). There was also a broad variation of cellularity of the tumors tested (tab. 1). The size of tumor population ranged from about 10^8 to 10^{11} cells (tab. 1). Assuming an exponential growth and a 100 % cell survival, the number of cell divisions underlying the size of the population was about 27 to 37 cell divisions (average: 31, tab. 1).

TRF Length Determination. When comparing the TRF lengths of uterine leiomyomas to the corresponding myometrium (fig. 3 and 4) an obvious decrease of TRF length was noted for all tumor samples analysed (tab. 1). For a more exact determination of the TRF length, all exposed X-ray-films were subjected to a densitometric evaluation (fig. 1). The 6 myometrial tissues had an average TRF length of about 13.7 kbp whereas the 19 uterine leiomyomas had an average TRF length of 11.2 kbp. The loss of TRF length in the tumors compared to the myometrial tissue ranged from 1120 bp to 4690 bp (average loss: 2560 bp, tab. 1).

No correlation between TRF loss and volume of the tumor ($r=0.0310$) was found. Analysis of correlation using only the five and six respectively tumor samples of patients 168 and 174 did not reveal a correlation as well ($r=0.3886$ and $r=0.1653$). There was also no

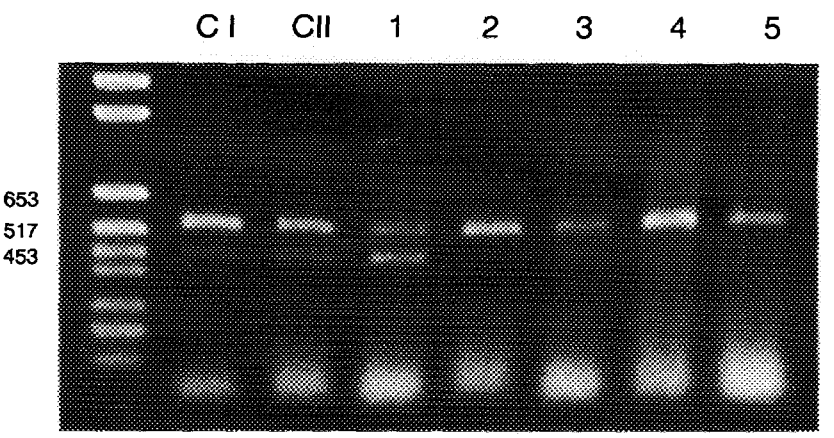

Figure 2. Clonal analysis of 5 uterine leiomyoma samples from the same patient showing heterozygosity for the PGK gene. Clonality was analysed as described by Noguchi et al. [28] by the loss of one of the two restriction fragments of the controls. CI and CII: control assays using the corresponding myometrium 174; 1-5: uterine leiomyomas 174.1, 174.2, 174.3, 174.4 and 174.5. The result of tumor 174.2 (2) is due to incomplete digestion of DNA since the analysis was repeated twice, each experiment showing loss of one band of the corresponding myometrial tissue.

Table 1: Summary of data obtained on 19 uterine leiomyoma samples and corresponding tissue

myometrial sample	tumor sample	average TRF length (bp)		loss of TRF length (bp)	cellularity area 1 (number of nuclei per 370000 µm²)	cellularity area 2 (number of nuclei per 370000 µm²)	average cellularity (number of nuclei per 370000 µm²)	volume of tumor (cm³)	size of tumor population (number of cells x 10⁶)	calculated number of cell divisions
161		13340								
	161.1		11260	2080	352	357	355	9,4	4,94	32
	161.2		10220	3120	217	133	175	7,9	2,05	30
	161.3		11430	1910	136	256	196	4,7	1,36	30
	161.4		10700	2640	299	244	272	3,9	1,57	30
	161.6		9760	3580	354	342	348	46,1	23,77	34
	161.7		11260	2080	279	226	253	11	4,12	31
174		13970								
	174.1		11610	2360	301	6	154	214,4	48,91	35
	174.2		11750	2220	72	86	79	5,6	0,66	29
	174.3		10550	3420	126	149	138	17,6	3,6	31
	174.4		11510	2460	111	184	148	8,9	1,95	30
	174.5		10570	3400	148	449	299	1,4	0,62	29
178		15190								
	178.1		14050	1140	-	-	-	4,7	-	-
	178.2		12200	2990	259	270	265	4,7	1,85	30
183		13360								
	183.1		8670	4690	275	260	268	3,1	1,23	30
	183.2		10150	3210	331	373	352	0,5	0,26	27
187		13860								
	187.3		12090	1770	219	365	292	3,9	1,69	30
	187.4		10770	3090	522	437	480	0,5	0,36	28
	187.5		12740	1120	417	405	411	0,8	0,49	28
193		12620								
	193.1		11300	1320	286	303	295	381,2	166,6	37
average:		13720	11190	2560			266	38	14,78	31

correlation between TRF loss and size of tumor population neither for all tumors (r=0.0483) nor for the tumor samples of patients 168 and 174 (r=0.3031 and r=0.0622). Based on an average number of 31 cell divisions and an average loss of 2560 bp we calculated an average loss of 83 bp of TRF per cell cycle.

DISCUSSION

Uterine leiomyomas are benign mesenchymal tumors mainly remaining asymptomatic [25]. Due to their histological characteristics uterine leiomyomas are an ideal system for the determination of TRF lengths.

Based on the monoclonal origin of the uterine leiomyomas and the total sizes of the tumor cell populations, we calculated an average loss of TRF length of approximately 83 bp/cell division which is in the range of the results reported in the literature. The decrease of TRF length in different types of cells has been calculated to be approximately 40 to 100 bp/MPD [3, 5, 6, 14, 27, 29].

The average length of TRF of the 6 myometrium samples was 13-14 kb. From the results of in vitro investigations it has been hypothesized, that the critical TRF length is reached at 4-5 kb [14, 30]. At this critical TRF size cells are assumed to enter crisis, which can be overcome only by cells expressing telomerase activity as a pre-requisite for their immortalization. Thus, we can calculate that the very first tumor cell of a uterine leiomyoma has to undergo approximately 100 cell divisions with an average loss of

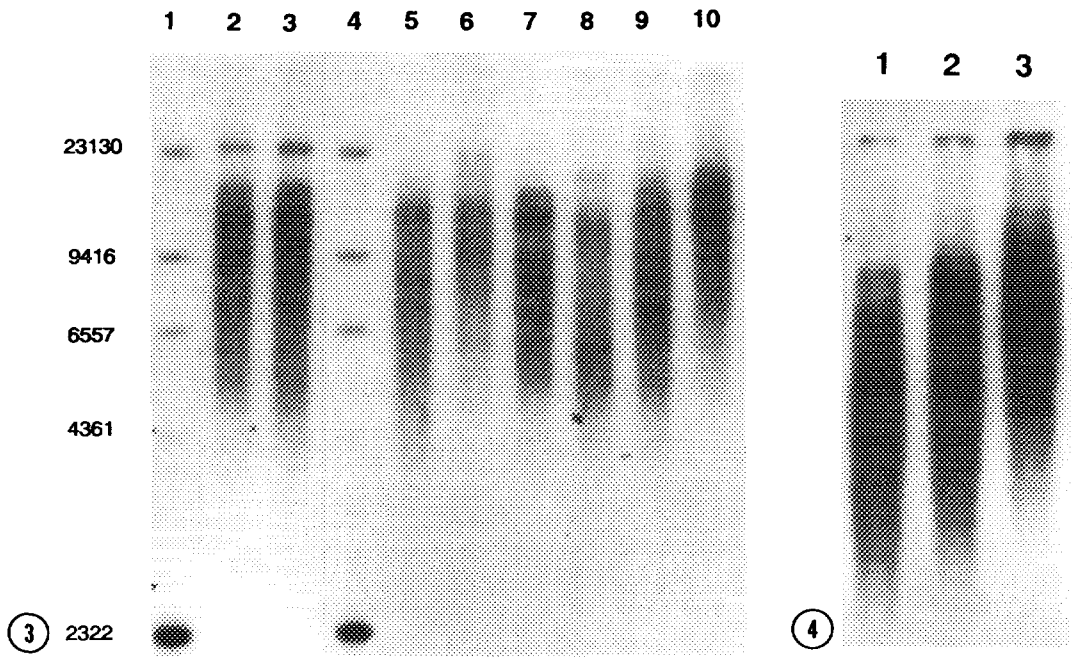

Figure 3. Southern blot analysis of TRF length of 6 uterine leiomyomas from one patient and the corresponding myometrium. Lane 1 and 4: DNA molecular weight standards; lane 2 and 3: uterine leomyoma 161.1; lane 5-9: uterine leiomyomas 161.2, 161.3, 161.4, 161.5 and 161.6; lane 10: myometrium 161.

Figure 4. Southern blot analysis of TRF length of 2 uterine leiomyomas from one patient and the corresponding myometrium. Lane 1 and 2: uterine leiomyomas 183.1 and 183.2; lane 3: myometrium 183.

approximately 85 bp per each cell division until the critical size of self-limiting growth is reached. Assuming an exponential growth and 100 % cell survival this would result in approximately 10^{30} cells, occupying a volume of 10^{21} cm^3 reflecting one part per million of the volume of the earth. Therefore, in uterine leiomyomas a telomerase activity is not necessary even for very large tumors.

The same can be concluded from the data reported by some authors: Transformed human embryonic kidney cells have to undergo in vitro approximately 75-120 MPD with an average loss of 65 bp/MPD until crisis is reached [29]. Transformed lymphocytes reach crisis in vitro after 60-100 MPD with an average loss of 100 bp/MPD [30]. Harley et al. [3] had shown that in fibroblasts a loss of 50 bp/MPD for 45-90 MPD occurs in vitro depending on the age or on the length of telomeric repeat of the donors [5]; in addition it is important that these aged cells are able to divide for further 20-30 times [31] until the critical TRF length is reached. Based on these data and taking a relatively low volume of 1000 μm^3 for a single tumor cell, a tumor size of approximately 10^{18} to 10^{36} cells can be revealed presenting tumor volumes ranging from 10^9 to 10^{27} cm^3 suggesting that also in

these cells telomerase activity is as a rule not a necessary prerequisite for a life-threatening growth of the tumor. This contradicts an extension of the telomere hypothesis to breast cancer as recently proposed by Shay et al. [13]. They assumed that reactivation of telomerase is a prerequisite for a tumor to grow to larger or even life-threatening sizes. However, we have to address to the point that telomerase activity has so far only been shown for malignant cell populations [14, 29] but not for any type of normal somatic tissues. The question arises whether or not there is just a coincidence between a poor differentation of cells and high levels of telomerase. Although the data presented herein do not allow for a definite answer yet, it must be pointed out that such a simple coincidence could also explain all data avaible so far. There is no definite proof yet that indeed immortalization of whatever cell population is causally related to expression of detectable levels of telomerase activity. As shown here the same holds true for the assumed correlation between a lack of telomerase activity and a self-limiting growth potential of tumors.

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