The hTERT mRNA in plasma samples of early breast cancer patients, non-cancer patients and healthy individuals

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One of the most important changes, which make cancer cells immortal, is reactivation of the telomerase enzyme. Human telomerase is composed of at least two subunits, hTERT and hTR. Many investigators have already detected telomerase mRNA in bodily fluids. The first aim of our study was to find out if there is a difference in the appearance frequency of detectable hTERT mRNA in plasma of early breast cancer patients, non-cancer patients and healthy individuals. The second aim was to determine whether surgical removal of the tumor affects the presence of hTERT mRNA in plasma of early breast cancer patients.

87 patients with early breast cancer, 22 non-cancer patients and 21 healthy individuals were included in the study. From early breast cancer patients, two blood samples were collected, the first prior and the second 24 hours after the surgical removal of the tumor. From other individuals one blood sample was collected. The presence or absence of hTERT mRNA was determined from all blood samples.

47% of early breast cancer patients, 32% of non-cancer patients and 5% of healthy individuals tested positive for the presence of hTERT mRNA in plasma. The difference between early breast cancer patients and healthy individuals was statistically significant (p<0,001). Among early breast cancer patients, 26% were positive for the presence of plasma hTERT mRNA before and after the surgical removal of the tumor, 21% were positive before and negative after, 36% were negative before and after and 17% were negative before and positive after the surgical removal of the tumor.

In conclusion, we found statistically significant difference of hTERT mRNA presence in plasma of early breast cancer patients when compared to healthy individuals. Second, we found that hTERT mRNA in plasma of early breast cancer patients is affected by the surgical removal of the tumor.

Key words: Breast cancer, breast surgery, telomerase, hTERT mRNA

Structural and numeric chromosomal aberrations are almost universal feature of cancers. In breast cancer, these changes seem to be an early and major driving force for the development of the disease [1]. This is supported by the results of some studies, which showed that these changes are present not only in invasive but also in ductal carcinoma in situ (DCIS) - a precursor of invasive breast cancer. Interestingly, the extensiveness of changes was proven to correlate with DCIS grade [2–4]. It is therefore important to identify mechanisms responsible for the evolution of chromosomal aberrations. Telomere shortening seems to be one of the most likely candidates [5].

Telomeres, repeating DNA sequences at the chromosomal ends, play a key role in structural and functional stabilization of chromosomes, which are shortened for 50-100 base pairs during each cell dividing process [5]. Consecutive cell divisions with resultant progressive telomere shortening lead to chromosomal instability [5]. Chromosomal instability drives the multiple genetic changes required for the formation of premalignant and malignant lesions. However, severe telomere shortening ultimately leads to lethal levels of genetic instability. Immortal cancer cells overcome this problem by reactivating an enzyme named telomerase [5]. Human telomerase, functioning as a telomeric DNA synthesizing enzyme, is composed of at least two subunits. The first subunit represents an RNA component (hTR), which serves as an RNA template for addition of specific telomeric repeats,

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Table 1. Patient and tumor characteristics.

		T1a and T1b tumors	T1c tumors	T2 tumors	T3 and T4 tumors
number of patients		12	47	21	7
mean age		58,7	59,6	60,9	65,2
grade	1 (%)	3 (25%)	15 (31,9%)	0 (0%)	2 (28,6%)
	2 (%)	6 (50%)	21 (44,7%)	10 (47,6%)	3 (42,9%)
	3 (%)	3 (25%)	11 (23,4%)	11 (52,4%)	2 (28,6%)
mean No of positive lymph nodes (range)		1,7 (0-12)	1,0 (0-33)	4,9 (0-25)	6,3 (0-24)
No of ER+ (%)[1]		9 (75%)	43 (91,5%)	15 (71,4%)	7 (100%)
No of HER-2 + (%)[2]		0 (0%)	2 (4,2%)	0 (0%)	0 (0%)
No of LVI + (%)[3]		1 (8%)	7 (14,2%)	5 (23,8%)	5 (71,4%)

[1] number of patients with positive ER status
[2] number of patients with positive HER-2 status
[3] number of patients with lymphovascular invasion

which are synthesized by the catalytic protein (hTERT), the second subunit of the telomerase [6, 7]. By reactivation of telomerase, cancer cells suppress further chromosomal instability, which would lead to cell death, and acquire the capacity for unlimited replication [8].

Unlike in normal tissues, where telomerase activity except for few exceptions (for example in lymphocytes and stem cells) is undetectable [9–12], approximately 85-95% of human cancers have been shown to express telomerase activity [13]. Like in other tumors telomerase activity is also present in the majority of breast carcinomas [14–18]. In most of breast tumors it has been shown that telomerase activity correlates with tumor progression, indicating at the same time more aggressive clinical behavior [19].

Taken together, telomerase activity seems to be a specific marker for cancer. However, its clinical utility depends on other factors such as the ability to detect it in bodily fluids [20]. Many investigators have already detected tumor derived RNA in the serum of cancer patients [20–27], including telomerase mRNA [20–22, 25, 26]. Since telomerase activity is regulated by the synthesis of hTERT mRNA, its presence in the bodily fluids would serve as an indicator of the whole telomerase activity in the human body [25]. Accordingly, we hypothesized that patients with breast cancer have higher levels of telomerase hTERT mRNA in bodily fluids than non-cancer patients and healthy individuals. Furthermore, we supposed that the presence of hTERT mRNA in plasma of early breast cancer patients is affected by the surgical removal of the tumor.

The first aim of our study was to find out whether there is a difference in the appearance frequency of detectable hTERT mRNA in plasma of early breast cancer patients, non-cancer patients and healthy individuals. The second aim was to determine whether surgical removal of the tumor affects the presence of hTERT mRNA in plasma of early breast cancer patients.

Materials and methods

Patients and blood sample collection. In the study we included 87 patients with early breast cancer, 22 patients with non-malignant diseases (various gastrointestinal diseases) and 21 healthy individuals. Mean age of the patients with early breast cancer and the pathological characteristics of the tumors are summarized in Table 1.

Two blood samples (10 ml each) were collected from all 87 patients with early breast cancer to determine the presence of hTERT mRNA in plasma. The first blood sample was collected prior the surgery on the day of the operation and the second blood sample was collected 24 hours after the surgical removal of the tumor.

One blood sample (10 ml) for plasma hTERT mRNA determination was collected from all of the non-cancer patients included in the present study during their hospital stay at the Gastroenterological clinic in Ljubljana.

One blood sample (10 ml) for plasma hTERT mRNA determination was also collected from all 21 healthy individuals.

All blood samples, that were collected to determine hTERT mRNA in plasma, were preserved in EDTA containing tubes (BD Vacutainer, Plymouth, UK). After the collection blood samples were immediately stored at 4°C until centrifuged at 880 x g for 10 minutes. The obtained plasma was centrifuged again at 880 x g for 10 minutes. Then the plasma was stored at -70°C until further processing took place.

RNA isolation. Before beginning the steps of RNA isolation, plasma samples were concentrated at 4°C in a concentrator (Eppendorf-Netheler-Hinz, Hamburg, D). Then TRIzol LS Reagent (Invitrogen, Life Technologies, Paisley, UK) was added to the concentrated plasma and homogenized samples were incubated for 5 minutes at room temperature. Chloroform was added next, followed by 15 seconds of shaking and 10 minutes of incubation at 4°C. The samples were then centrifuged at 12000 x g for 15 minutes at 4°C. The upper aqueous phase (that contains RNA) was carefully transferred into a fresh tube and incubated for 16 hours at -20°C with isopropyl alcohol, 3 mM Na-acetate and with RNase-free glycogen to allow the RNA precipitation. The mixture was then centrifuged at 12000 x g for 30 minutes at 4°C and the supernatant removed, while the pellet was washed by centrifugation (7500 x g for 5minutes at 4°C) with 75% ethanol. Finally, the supernatant was dis-

Table 2. Non-cancer patient ID, age at the sample collection, sex, diagnosis and presence (+) or absence (-) of the hTERT mRNA in plasma.

ID	age	sex	diagnosis	hTERT mRNA	lymphocyte count (*10^9/l)
1	76	M	sepsis	+	1,1
2	59	M	sepsis	-	0,3
3	58	F	st. post liver transplantation	-	NA
4	46	M	st. post liver transplantation	+	0,9
5	76	M	ethylic liver disease	-	0,9
6	44	M	ethylic liver disease	+	NA
7	71	M	ethylic liver disease	-	0,3
8	50	M	ethylic liver disease	-	1,3
9	71	F	mb. Crohn	-	1,7
10	47	F	mb. Crohn	-	1,1
11	49	M	mb. Crohn	+	2,0
12	61	F	acute pancreatitis	-	NA
13	32	M	acute pancreatitis	-	NA
14	39	M	acute pancreatitis	-	NA
15	73	F	esophagitis	+	NA
16	59	M	esophagitis	-	NA
17	77	M	toxic liver damage	-	0,2
18	73	F	stenosis of the billiary tract	-	NA
19	37	F	gastroenterocolitis	-	1,3
20	70	F	gastrointestinal bleeding	+	NA
21	80	M	diarrhea	-	1,1
22	68	F	cholecystolithiasis	+	0,6

NA – not available

carded and RNA pellet was dried for 5-10 minutes at room temperature, dissolved at 60°C in 15 µl of DEPC-treated water and stored on ice.

RNA amplification. After RNA isolation, reverse transcription (RT) of RNA to cDNA was performed and then cDNA fragments were amplified by PCR reaction. Both steps were performed using a GeneAmp RNA PCR Core Kit (Applied Biosysems, Roche Molecular Systems, Branchburg, NJ, USA) according to the manufacturer's instructions. The amplification primer was 5'-TGA CAC CTC ACC TCA CCC AC-3' for hTERT-sense and 5'-CAC TGT CTT CCG CAA GTT CAC-3' for hTERT-antisense. The final volume of reaction mixture in the first step was 20 µl, containing 5 mM $MgCl_2$, 1X PCR buffer II, 1 mM of each deoxynucleotide triphosphate, 1U/µl RNase inhibitor, 2.5 U/µl MuLV reverse transcriptase, 2.5 µM random hexamers and sample RNA. The PCR amplification was performed in the same reaction tube as the RT, by adding new reagents and adjusting the final concentration for all reagents in the reaction mixture. The final mixture volume was thus increased to 100 µl consisting of 1X PCR buffer II, 2.5 U AmpliTaq DNA polymerase, 2.3 mM of $MgCl_2$ and 0.5 µM of primers (sense and antisense). The PCR conditions were as follows: an initial incubation at 95°C for 2 minutes followed by 35 cycles at 95°C (30 seconds), 60°C for 1 minute, 72°C (30 seconds), and final elongation at 72°C for 7 minutes.

PCR products analysis. PCR products were analyzed by capillary electrophoresis using the bioanalyzer and a DNA 1000 Assay Kit (Agilent Tecnologies, Waldbronn, D), according to the manufacturer's instructions. The method allowed the determination of size (in bp) and concentration (ng/µl) of PCR products. Briefly, 1 µl of sample was added into the sample well loaded with gel-dye mix and buffer. The chip was vortexed for 1 minute and placed in the bioanalyzer. After chip run, the samples (PCR products) moved through the micro-channels and were electrophoretically separated. The fluorescence of PCR products was detected and translated into electropherograms.

Statistical analysis Descriptive statistics of patient and tumor characteristics was performed. Proportion of early breast cancer patients, non-cancer patients and healthy individuals that tested positive for the presence of hTERT mRNA in plasma was compared using two sided exact Pearson's chi square. The average lymphocyte count in non-cancer patients with positive and negative plasma hTERT mRNA was compared using two-sided t test. Early breast cancer patients were divided into four groups with regard to the presence of hTERT mRNA before and after the surgical removal of the tumor.

Results

Appearance of hTERT mRNA in plasma of early breast cancer patients, non-cancer patients and healthy individuals. Among early breast cancer patients were 41/87 (47%) tested positive for the presence of hTERT mRNA in plasma (mean age 60.3). Among patients with various gastrointestinal diseases (non-cancer patients) 7/22 (32%) were tested positive for the presence of hTERT mRNA (mean age 59.8) and finally, among healthy individuals 1/21 (5%) were tested positive for the presence of hTERT mRNA in plasma (mean

Table 3. Number of patients with regard to the presence of hTERT mRNA before and after the surgical removal of the tumor.

		after surgical removal of the tumor	
		hTERT +	hTERT -
before surgical removal of the tumor	hTERT +	23 (26%) *group I*	18 (21%) *group II*
	hTERT -	15 (17%) *group IV*	31 (36%) *group III*

age 35.6 years). When early breast cancer patients were compared with other two groups, we found statistically significant difference between the early breast cancer patients and healthy individuals (two sided exact Pearson's chi square; p<0,001), but not statistically significant difference between early breast cancer patients and non-cancer patients (two-sided exact Pearson's chi square; p=0.235).

Results of hTERT mRNA measurement in non-cancer patients, their lymphocyte count, diagnosis and some demographic characteristics are shown in Table 2. We found no relationship between the specific diagnosis and the presence of hTERT mRNA in plasma of these patients. The average lymphocyte count in patients with positive and negative hTERT mRNA in plasma was 1.2 and $0.9*10^9$/l, respectively. The difference is not statistically significant (two-sided t-test; p=0.523).

Influence of the surgical removal of the tumor on the presence of hTERT mRNA in plasma of early breast cancer patients. There were 87 early breast cancer patients included in the study. All patients were women with the mean age 60.3 years. The patients were divided into four groups with regard to the presence of the plasma hTERT mRNA before and after the surgical removal of the tumor (Table 3). Before surgical removal of the tumor there were 41/87 (47%) patients positive and 46/87 (53%) negative for the presence of hTERT mRNA in plasma. After the surgical removal of the tumor hTERT mRNA was again detected in 23 (56%), while in 18 of 41 (44%) patients that were positive before the surgical removal of the tumor, the hTERT mRNA was no more detectable. Additionally, after the surgical removal of the tumor the hTERT mRNA was detected in 15 of 46 (33%) patients that were hTERT mRNA negative before surgical removal of the tumor.

Discussion

When comparing the proportion of individuals tested positive for hTERT mRNA in plasma of early breast cancer patients, non-cancer patients and healthy individuals, we found statistically significant difference between early breast cancer patients (47%) and healthy individuals (5%). The proportion of non-cancer patients that tested positive for hTERT mRNA in plasma was 32% and the difference between this group and early breast cancer patients was not statistically significant. These results indicate that hTERT mRNA may be detectable in plasma samples even of patients with various non-malignant diseases. We supposed that this might be the result of inflammation followed by an increased number of lymphocytes that are known to express telomerase enzyme. However, we found lymphocyte count in those patients rather low and never above the upper limit. Furthermore, when the mean lymphocyte count was compared between the patients that were hTERT mRNA positive with those that were negative, there was no statistically significant difference. Accordingly, there should be other reasons for elevated hTERT mRNA levels in those patients. As stem cells are also known to express telomerase enzyme, we suppose more extensive regeneration of tissues through accelerated stem cell division and differentiation as one of the possible reasons. Other possibility for higher proportion of non-cancer patients tested positive for hTERT mRNA in plasma compared to healthy individuals might be age difference.

To answer the question whether the surgical removal of the tumor affects the presence of hTERT mRNA in plasma of early breast cancer patients, we divided the patients into four groups. First group represents patients that were positive before and after the surgical removal of the tumor (26%). Second group represents patients that were positive before and negative after the surgical removal of the tumor (21%). Besides first two groups of patients there were a substantial number of patients that were both times negative (36%) (the third group) and patients that were negative before and positive after the surgical removal of the tumor (17%) (the fourth group).

Among 41 patients that were positive before the surgical removal of the tumor (the first and second group) 23 (56%) tested positive (the first group) and 18 (44%) tested negative (the second group) for the presence of hTERT mRNA in plasma 24 hours after the surgical removal of the tumor. Of note, during the operation the removal of all known tumor burden was performed. As a consequence, the main telomerase expressing tissue was removed. Therefore, we assumed that surgical removal of the tumor would resulted in a drop of the level of hTERT mRNA in plasma to the undetectable levels. Indeed, we observed this drop in almost 50% of cases.

However, the hTERT mRNA in plasma of the other half of the patients did not resulted in the same drop. Those patients may have a micrometastatic disease and/or a greater amount of circulating tumor cells with high expression of hTERT mRNA, which can represent a continual source of hTERT mRNA in plasma. In this scenario, the presence of hTERT mRNA in plasma after the surgical removal of the tumor may indicate a more aggressive biological behavior of the tumor and thus worse prognosis.

Besides first two groups of patients that were positive before the surgical removal of the tumor, there were more than 50% of early breast cancer patients that were negative for the presence of hTERT mRNA in plasma before the surgical re-

moval of the tumor (the third and fourth group). In these patients small tumor burden and possible specific pathological characteristic of the tumor (less necrosis, apoptosis and invading of tumor cells into the bloodstream) might be the reason for undetectable levels of hTERT mRNA in plasma before the surgical removal of the tumor. All of this might again have prognostic and predictive significance. In the fourth group of patients (negative before and positive after the surgical removal of the tumor) the increase of the hTERT mRNA in plasma after the surgical removal of the tumor can be explained with tumor manipulation during surgery, which was shown to increase the level of circulating breast cancer cells after the operation [28]. However, it was also shown that these tumor cells were not related to bad prognosis [28]. Accordingly, we can expect the worst prognosis in the first group of patients and the most favorable prognosis in the third and fourth group of patients with the second group somewhere in-between. A prospective study would be necessary to perform to find out the meaning of the plasma hTERT mRNA as a prognostic factor in early breast cancer patients.

In conclusion, we found statistically significant difference of hTERT mRNA presence in plasma of early breast cancer patients when compared to healthy individuals. When comparing early breast cancer patients with non-cancer patients this difference was not statistically significant. Second, we found that hTERT mRNA in plasma of early breast cancer patients is affected by the surgical removal of the tumor. The clinical applications of our findings are not yet known.

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