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(54) **DETECTION D'UN CARCINOME DANS LA VESSIE A PARTIR D'UN ECHANTILLON D'URINE**

(54) **DETECTION OF URINARY BLADDER CARCINOMA IN A URINE SAMPLE**

(57) The present invention concerns a method for the detection of urinary bladder carcinoma in a urine sample as well as suitable reagents therefor.

Abstract

The present invention concerns a method for the detection of urinary bladder carcinoma in a urine sample as well as suitable reagents therefor.

Detection of urinary bladder carcinoma in a urine sample

Description

The present invention concerns a method for the detection of urinary bladder carcinoma cells in a urine sample as well as reagents suitable therefor.

Urothelial carcinoma of the urinary bladder is the most frequent malignant tumour of the urinary tract and occurs in ca. 15 of 100,000 persons. At present the detection of urinary bladder carcinomas requires invasive cytoscopic examinations. A reliable non-invasive method for detecting urothelial carcinomas of the urinary bladder would open up new opportunities for the diagnosis as well as the aftercare of patients with urinary bladder carcinomas. In addition such a method would be useful for screening persons from risk groups such as smokers or workers in the chemical industry.

Human telomerase is a ribonucleoprotein which can cause a de novo synthesis of telomer ends. Telomers are specific structures at the ends of chromosomes and in eukaryotic organisms are composed of many repetitions of short sequences e.g. the sequence TTA GGG in humans. In somatic cells each replication of the cells inevitably leads to a shortening of the telomer ends and, when the telomer falls below a certain length, it finally leads to death of the cell.

In contrast virus-transformed or immortalized cells such as tumour cells exhibit no reduction in telomer length. This is due to the activity of telomerase. Since,

according to previous findings, the expression of telomerase is restricted to tumour cells, germ cells and immortalized cells, it is a promising parameter for the diagnosis of tumours as well as a point of attack for tumour therapy.

The structure of the RNA domains of the gene coding for telomerase was recently determined in the mouse (Blasco et al., Science 269 (1995), 1267-1270) and in humans (Feng et al., Science 269 (1995), 1236-1241). The gene for human telomerase RNA (hTR) is located on chromosome 3 section q 26.3 (Soder et al., Oncogene 14 (1997), 1013-1021; Parkinson et al., Eur. J. Cancer 33 (1997), 727-734). The protein domains of the telomerase from various lower eukaryotes are also known (Collins et al., Cell 81 (1995), 677-686). An mRNA coding for a homologous protein whose expression correlates with telomerase activity has recently been discovered in humans (Nakamura et al., Science 277 (1997), 955-969; Meyerson et al., Cell 90 (1997), 785-795).

Methods for the detection of urinary bladder carcinoma by determining the telomerase activity are known (Müller et al., Int. J. Oncol. 9 (1996), 1169-1173; Yoshida et al., Cancer 79 (1997), 362-369; Lin et al., Clin. Cancer Res. 2 (1996), 929-932 and Kinoshita et al., J. Natl. Cancer Inst. 89 (1997), 724-730; Dalbagni et al., Clinic. Cancer Res. (1997), 1593-1598; WO97/35871).

Lin et al. describe the detection of telomerase activity in tissue samples from patients with urinary bladder carcinoma. A sensitivity of 97.5 % was found. However, this method has the disadvantage that the sample can only be obtained by invasive measures so that it is not

suitable for an application in the clinical routine.

Müller et al. describe the detection of telomerase activity in tissue samples and bladder rinses from urinary bladder carcinoma patients. No telomerase activity was detected in the urine of patients with urinary bladder carcinoma. Yoshida et al. describe the detection of telomerase activity in the urine of urinary bladder patients. A test sensitivity of 62 % was found. Kinoshita et al. also describe the detection of telomerase activity in urine samples in which a sensitivity of 55 % is found. Dalbagni et al. describe the detection of telomerase activity in urine samples. The sensitivity of the test was 18 to 50 % depending on the respective tumour stage.

WO97/35871 describes a method for the detection of urinary bladder carcinoma cells in a urine sample by determining the telomerase activity. A sensitivity of 87 % is described when the sample is directly processed further without freezing.

With regard to this prior art it is apparent that, although telomerase activity is a potentially useful parameter for detecting urinary bladder carcinoma, previous non-invasive methods have not given reliable results. Results that differ greatly from one another have been obtained by the various authors especially when detecting telomerase activity in urine samples which indicates major problems with regard to reproducibility.

Hence the object of the present invention was to provide a new non-invasive method for the detection of urinary

bladder carcinomas in which the disadvantages of the prior art are at least partially eliminated. In particular the method should have a high sensitivity and reproducibility and, moreover, be suitable for routine diagnostic applications also with regard to sample collection and preparation.

This object is achieved by a method for the detection of urinary bladder carcinoma in a urine sample wherein
(a) a urine sample is prepared and
(b) telomerase RNA is determined in the sample.

Surprisingly the method according to the invention enables a sensitive and reproducible detection of telomerase RNA in a sample as aggressive as urine in which an acidic pH value, RNases, salts and urea are present. A comparison of the method according to the invention with prior art methods for the determination of the enzymatic telomerase activity shows that the determination according to the invention of telomerase RNA has significant advantages with regard to sensitivity and reliability with the same type of sample preparation.

The presence of telomerase RNA in the sample is an indicator for the presence of a urinary bladder carcinoma especially if the amount of telomerase RNA is above a predetermined threshold value.

Step (a) of the method according to the invention comprises preparing a urine sample. This urine sample is preferably derived from a mammal. It is particularly preferably a human urine sample. Step (a) preferably comprises isolating cells from the urine e.g. by

centrifugation and lysing them. This lysis can for example be carried out by detergent extraction in which the cells are treated with an extraction buffer which contains 0.01 to 5 % by weight of a non-ionic or zwitterionic detergent such as CHAPS. On the other hand the cell lysis can also comprise an RNA enrichment e.g. with the commercially available RNAzol kit. Both lysis methods yield comparable results. Cell lysis by means of heat denaturation is also possible.

The cells are preferably lysed in the presence of an RNase inhibitor such as RNAsin. After the lysis the sample can be frozen and rethawed for the determination. The freezing is preferably carried out by shock freezing in liquid nitrogen. After freezing the sample is stable for several weeks or months at a temperature of -80°C.

However, it is not necessary to freeze the sample. It was surprisingly found that telomerase RNA can also be reliably detected without processing the urine after storage for several days at 4°C or at room temperature. This is an unexpected finding since the enzymatic activity of telomerase RNA in a urine sample is already no longer detectable after only a very short storage period. This surprising sample stability is a prerequisite for a routine application in diagnostics since sample collection and sample preparation are usually carried out at different sites by different persons.

Step (b) of the method according to the invention comprises the determination of a telomerase RNA, preferably the determination of human telomerase RNA. This determination can be carried out without marker

groups, with radioactive marker groups or with non-radioactive marker groups. Non-radioactive marker groups are preferably used. All known marker groups can be used as non-radioactive marker groups e.g. immunologically reactive groups such as nucleotide analogues or haptens which can react with a detection antibody, enzymes such as peroxidase, galactosidase or alkaline phosphatase, fluorescent groups or luminescent groups such as chemiluminescent or electrochemiluminescent groups, donor-acceptor systems or other detection groups such as NMR-active marker groups or electron-dense groups. Immunologically reactive groups are preferred such as nucleotide analogues e.g. halogen-derivatized nucleotides such as Br-dUTP or nucleotides derivatized with organic residues which contain at least one C atom such as CH_3-dCTP, or haptens such as digoxigenin, digoxin, fluorescein etc., luminescent groups such as acridinium ester or luminescent metal complexes e.g. ruthenium complexes and fluorescent groups such as fluorescein and donor-acceptor systems such as fluorescence resonance energy transfer systems.

The determination of telomerase RNA according to step (b) of the method according to the invention preferably comprises a reverse transcription step in which the telomerase RNA is transcribed into a complementary DNA. In addition the determination also preferably includes a nucleic acid amplification step. The type of amplification step is uncritical for the method according to the invention. Typically the amplification is carried out by adding a suitable enzyme which can polymerize nucleic acids such as a nucleic acid polymerase or a nucleic acid ligase. It is preferable to use a thermostable enzyme and to carry out the amplification in several cycles. The enzyme is

particularly preferably a thermostable DNA polymerase e.g. Taq polymerase. A particularly preferred amplification method is the polymerase chain reaction (PCR). The determination according to the invention of human telomerase RNA particularly preferably comprises a combination of reverse transcription and PCR.

Two primers are preferably used for the amplification which are complementary to telomerase RNA or to a cDNA synthesized therefrom. In the case of human telomerase RNA, suitable primer sequences can be selected on the basis of the nucleotide sequence stated in Feng et al., supra. For example a primer pair can be used for the nucleic acid amplification selected from :

(a) nucleotides with the nucleotide sequences shown in SEQ ID NO. 1 and SEQ ID NO.2 and

(b) nucleotides which contain a partial sequence of the nucleotide sequences shown in SEQ ID NO.1 and SEQ ID NO.2 having a length of at least 5 nucleotides.

The telomerase RNA can be detected qualitatively or quantitatively by the method according to the invention. A test which results in an amplification product derived from telomerase RNA which is in particular above a predetermined threshold value is rated as positive. The threshold value is preferably specified such that the weak signals that occasionally occur in normal patients are classified as negative and the strong signals derived from patients with urinary bladder carcinoma are classified as positive. A quantitative detection of telomerase RNA enables conclusions to be drawn about the degree of differentiation or the stage of carcinomas since the signal strength increases with the aggressiveness of the carcinoma.

On the one hand, the detection can comprise a separation of the reaction mixture according to size e.g. a separation in a non-denaturing agarose gel. In addition to size, other properties of the amplification products can also be used for the differentiation e.g. the presence/absence of a restriction enzyme cleavage site, melting temperature of the amplification products etc.

Alternatively the amplification products obtained by the method according to the invention can also be analysed without prior separation of the reaction mixture e.g. after immobilization on a solid phase or by on-line detection in a homogeneous test format. The wall of a reaction vessel can for example serve as a solid phase. In addition it is also possible to use particulate solid phases. The solid phase is preferably selected from microtitre plates, microreaction vessels, membranes, microchips, biocore systems and optionally magnetic microbeads. An advantage of immobilization is the large saving in time and small amount of manual work. Furthermore a high sample throughput and automation, e.g. for routine analytics, are possible.

The amplification products can, in principle, be immobilized on a solid phase by any known method e.g. by adsorptive binding. Preferably the immobilization is achieved by specific interactions e.g. via anchor groups. Examples of suitable anchor groups are immunologically reactive groups which can react with a solid phase-bound antibody, or other groups which are able to bind with high affinity to an immobilized partner. A preferred example of an anchor group is biotin which can bind with high affinity to an avidin or streptavidin-coated solid phase.

An on-line detection can for example be carried out by donor-acceptor detection systems or by acridinium ester detection groups in a homogeneous detection method. An appropriate test format is described by Arnold Jr. et al. (Clin. Chem. 35 (1989), 1588-1594).

The marker or anchor groups can be introduced into the amplification product at various stages of the method according to the invention. Thus, for example, one or several primers can be used which already contain marker groups or/and anchor groups. On the other hand labelled nucleotides can also be introduced into the amplification product during the amplification.

However, in a preferred embodiment of the present invention it is not necessary at all that the amplification product itself contains an anchor or/and marker group. These groups can also be present in one or several hybridization probes which are able to hybridize in a stable manner with the amplification product under the test conditions and in this manner enable a specific detection. Anchoring to the solid phase can for example be achieved by adding one or several capture probes which carry one or several anchor groups. Marker probes can also be used in a corresponding manner to detect the amplification product. Examples of suitable hybridization probes are oligonucleotides which contain an anchor or marker group at their 5' or 3' end. On the other hand it is also possible to use nucleic acid analogues as hybridization probes e.g. peptidic nucleic acids (Nielsen et al., Science 254 (1991), 1497-1500 and Dueholm et al., J. Org. Chem. 59 (1994), 5767-5773).

The telomerase RNA is preferably detected in a known

manner by means of the marker groups contained in the amplification product or by means of the marker probes bound to the amplification product. The detection is preferably achieved by using non-radioactive marker groups in automated measurement devices. Measurement devices are preferred in which the marker groups are detected by colorimetric or/and spectrophotometric methods e.g. by enzymatic conversion of a substrate or by chemiluminescence or fluorescence.

The method according to the invention is preferably carried out in such a way that a standard nucleic acid and in particular an RNA which is different from telomerase RNA is determined in the sample in addition to the determination of telomerase RNA. Nucleic acid-like molecules e.g. nucleic acid analogues can also be used as a standard nucleic acid.

The standard is preferably an RNA which is constitutively expressed by the respective organism e.g. a human rRNA in particular 28S rRNA, β2-microglobulin mRNA or GAPDH-mRNA. Alternatively a nucleic acid, in particular a RNA, added to the sample from outside e.g. before the sample preparation or before the amplification can also be used as a standard. Moreover a modified telomerase RNA can be used as a standard which can be differentiated from the telomerase RNA to be detected on the basis of one or several characteristics such as a mutation of the sequence e.g. by insertion or/and deletion of one or several nucleotide building blocks. The standard is preferably determined in an analogous manner to the determination of the human telomerase RNA and can for example comprise a reverse transcription or/and a nucleic acid amplification. The standard is particularly preferably determined by using a combination of reverse

transcription and PCR:

If human β2-microglobulin RNA is determined as a standard, the use of a primer pair in the nucleic acid amplification has proven to be suitable which is selected from:

(a) nucleotides with the nucleotide sequences shown in SEQ ID NO. 3 and SEQ ID NO.4 and

(b) nucleotides which contain a partial sequence of the nucleotide sequences shown in SEQ ID NO.3 and SEQ ID NO.4 with a length of at least 5 nucleotides.

The method according to the invention can be carried out as a one-pot reaction especially if the telomerase RNA and optionally the standard is detected under conditions that allow a specific detection of the telomerase RNA and - if present - the standard without separation of amplification by-products.

This specific detection can be achieved in a simple manner when the amplification products are separated electrophoretically by determining one band with a characteristic size in the electrophoresis medium. If a standard is determined in parallel, it is expedient to use a primer pair in this case which on amplification yields a product with a size that can be clearly distinguished from the size of the human telomerase RNA amplification product. A specific detection is also possible by means of other properties of the amplification products in a corresponding manner.

When analysing the products of the amplification mixture without prior separation e.g. by immobilization or by on-line detection, differentiation between human telomerase

RNA, standard and optionally amplification by-products can be carried out by various measures e.g. by using different labels or/and anchor groups (e.g. primers, capture probes or/and marker probes each with different groups), by selecting hybridization conditions which allow such a specific detection or/and by immobilizing the standard and the telomerase RNA amplification product at different sites on the solid phase or on different solid phases. In addition the specificity can be improved by adding unlabelled oligonucleotides or nucleic acid analogues (competitors) which are complementary to the primer sequences so that these sequence regions are masked and the hybridization with the capture or detection probe occurs specifically on internal sequences of the amplification products.

Yet a further subject matter of the present invention is a reagent kit for the detection of a telomerase RNA comprising:

(a) a primer for the reverse transcription of a telomerase RNA
(b) an agent for reverse transcription,
(c) a primer pair for the amplification of a DNA obtained from telomerase RNA by reverse transcription,
(d) an agent for nucleic acid amplification and
(e) marker groups e.g. non-radioactive marker groups.

The marker groups can be present in the form of appropriately labelled nucleoside triphosphates or/and labelled primers. On the other hand the marker groups can also be present on one or several marker probes which hybridize with the amplification product under the test conditions.

The reagent kit can additionally contain solid phase anchor groups and a solid phase. The solid phase anchor groups are preferably biotin and the solid phase is coated with streptavidin or/and avidin. The solid phase anchor groups can be present in the form of modified nucleoside triphosphates or be present on a primer. Alternatively the solid phase anchor groups can also be present on a capture probe which hybridizes with the amplification product.

Alternatively the reagent kit can contain detection groups and optionally hybridization probes which enable an on-line detection in particular in a homogeneous test format.

The agents for reverse transcription and nucleic acid amplification preferably comprise enzymes, nucleoside triphosphates and optionally buffer substances that are suitable for the respective purpose.

Furthermore the reagent kit according to the invention can contain a primer for the reverse transcription of a standard nucleic acid which is different from telomerase RNA and a primer pair for amplifying a DNA obtained from the standard by reverse transcription.

In a particularly preferred embodiment the reagent kit contains one or several hybridization probes e.g. marker or/and capture probes which contain marker or/and solid phase anchor groups and which hybridize with the amplification product or, if using a standard with its amplification products. The marker and capture probes are preferably selected from oligonucleotides and nucleic acid analogues in particular peptidic nucleic acids.

In addition the reagent kit can also contain positive or/and negative controls as well as an internal standard for quantifying the detection reaction and corresponding agents for the separate detection of the standard and amplification product.

The reagent kit according to the invention is especially suitable for use in a non-invasive method for the detection of urinary bladder carcinomas e.g. in a method as described above.

The invention is further elucidated by the following examples and sequence protocols:

SEQ ID NO.1 and 2	show amplification primers for determining human telomerase RNA,
SEQ ID NO.3 and 4	show amplification primers for determining β2-microglobulin mRNA as a standard and
SEQ ID NO.5 and 6	show primers for determining telomerase activity.

Examples

1. Collection and pretreatment of urine samples

Samples of in each case 100 ml of the second morning urine were collected from 30 patients with confirmed urothelial carcinoma of the urinary bladder, 15 patients with other urological diseases (urolithiasis, infections of the urinary tract), 3 patients in aftercare for urinary bladder carcinoma and 20 healthy volunteers.

All samples were processed further within 15 min. The samples were divided into 2 aliquots of 50 ml. Both aliquots were centrifuged and washed twice with PBS after which one sample was subjected to a CHAPS extraction and the other to an RNAzol extraction.

For the CHAPS extraction the cell pellets obtained from the centrifuged urine were lysed by the method of Kim et al., (Science 266 (1994), 2011-2015) using 200 µl lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercapto-ethanol, 0.5 % CHAPS, 10 % glycerol). The lysate was then shock-frozen in liquid nitrogen and stored at -80°C.

The RNAzol extraction of the pellets obtained from the centrifuged urine was carried out according to the manufacturer's instructions (WAK-Chemie-Medical GmbH, Bad Homburg, Germany). The lysate was shock-frozen in liquid nitrogen and stored at -80°C.

2. Telomerase detection method

2.1 Detection of telomerase activity (TRAP assay)

The TRAP assay was carried out according to the method described by Kim et al. (supra). The protein content of the sample was determined using the Biuret method. 10 µg protein concentrate, 20 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005 % Tween-20, 1 mM EGTA, 50 µM deoxynucleotide triphosphate, 0.1 µg oligonucleotide TS (SEQ ID NO 5: 5'-AAT CCG TCG AGC AGA GTT-3') 1 µg T4g32 protein (Boehringer Mannheim) and 0.1 mg/ml bovine serum albumin were incubated for 30 minutes at 35°C. Samples incubated with 1 µg RNAse and blank samples containing

lysis buffer or H_2O were examined as negative controls.

For the amplification 2.5 U Taq-DNA polymerase (Perkin-Elmer, Weiterstadt, Germany) and 0.1 µg oligonucleotide Cx (SEQ ID NO. 6: 3'-AAT CCC ATT CCC ATT CCC ATT CCC-5') were added to 50 µl sample volume. 30 PCR cycles (94°C for 30 s, 50°C for 30 s and 72°C for 90 s) were carried out in a thermocycler (TC9600, Perkin-Elmer, Weiterstadt, Germany).

The PCR products were detected by non-radioactive labelling. For this the Cx primer was labelled with the fluorescent dye FAM (Perkin-Elmer, Weiterstadt, Germany). Fluorescent amplification products were detected after electrophoresis in a 4.5 % polyacrylamide/6 M urea sequencing gel on an automated sequenator 377 using the GeneScan Software package (Perkin-Elmer, Weiterstadt, Germany).

In parallel the telomerase PCR-ELISA kit (Boehringer Mannheim, Mannheim, Germany) was used to detect non-radioactive PCR products using a biotin-labelled TS primer.

2.2 Detection of human telomerase RNA (hTR) by RT-PCR

2 µg total RNA from the RNAzol extraction or 2 µg total protein from the CHAPS extraction were used to carry out the RT-PCR. The PCR was carried out after adding 23 µl of a reaction mixture which contained the reagents from the Titan™ One Tube RT PCR system (Boehringer Mannheim) plus in each case 150 µM of the primer pairs specified in the following. After a denaturation step (6 min at 94°C) and reverse transcription (30 min at 65°C) an additional

short denaturing step and 25 cycles of a PCR (94°C for 30 s and 65°C for 1 min, extension: 60°C for 7 min) were carried out.

In order to detect the telomerase RNA the primer pair TE-hTR 5.3 (SEQ ID NO.1: 5'-AAC TGA GAA GGG CGT AGG CGC CGT GC-3') and TE-hTR 3.1 (SEQ ID NO.2: 5'-GTT TGC TCT AGA ATG AAC GGT GGA AG-3') were used to obtain a 111 bp long amplification product. The primer pair TE-β2M 5.2 (SEQ ID NO.3: 5'-TTC ACC CCC ACT GAA AAA GAT GA-3') and (SEQ ID NO.4: 5'-GGC ATC TTC AAA CCT CCA TGA TG-3') were used as an internal standard to detect β2-microglobulin mRNA which resulted in a product of 119 bp. For the non-radioactive detection of the PCR products, one of the primers in each case (TE-hTR5.3 or TE-β2M5.2) was labelled with FAM (Perkin-Elmer Company). The electrophoresis and detection of the PCR products was carried out as described under item 2.1.

Human tissue samples from a urinary bladder carcinoma and samples of cells from a human urinary bladder carcinoma cell line J82 (ATCC HTB1) were used as positive controls. This cell line was cultured in DMEM containing 10 % foetal calf serum, 2 % L-glutamine, 1 % penicillin streptomycin (Gibco).

3. Results

Large amounts of human telomerase RNA (hTR) were detected in J82 cells and tissue samples from a bladder carcinoma in the CHAPS lysate as well as in RNA preparations obtained by RNAzol extraction. A high telomerase activity was also found in these samples. Only samples with distinct bands of 119 bp (presence of the β2-micro-

globulin mRNA) and 111 bp (presence of hTR) were classified as positive with regard to the determination of hTR. Samples with a band at 119 bp and without a band at 111 bp (absence of hTR) were classified as negative.

The TRAP assay of telomerase-positive samples showed the characteristic 6 bp ladder after GeneScan analysis. In a telomerase PCR ELISA, telomerase-positive samples exhibited absorptions (A_{450}-A_{690}) between 250 and 2,000. Samples treated with RNase exhibited no telomerase activity. Blank samples (lysis buffer or water) also exhibited no telomerase activity. In the telomerase PCR ELISA telomerase-negative samples and controls exhibited an absorption of less than 100. Both methods for the detection of TRAP products (GeneScan analysis or telomerase PCR ELISA) yielded comparable results.

Detectable amounts of hTR were found using RT-PCR in 25 of 30 urine samples (82 %) which were derived from patients with confirmed urinary bladder carcinoma. Telomerase activity was only found in two cases (7%). hTR was detected by RT-PCR in 3 of 20 samples (15 %) from healthy volunteers.

In patients without malignant diseases but with known benign urological diseases such as urolithiasis or infections of the urinary tract, hTR was found in 4 of a total of 15 samples (27 %). In one female patient with a urinary tract infection and an initially detectable hTR, hTR was no longer detected after treating the infection. Telomerase activity was detected in none of the patients.

Samples of three patients were examined during aftercare for urinary bladder carcinomas. hTR or telomerase

activity was detected in none of these patients in which no renewed occurrence of the malignant disease was found.

The results are summarized in the following Tables.

Table 1 shows the clinical pathological classification and the results of the detection of hTR and telomerase activity in urine samples from patients with histologically confirmed urinary bladder carcinoma.

Table 1

Patient	Stage	Degree	telomerase activity	hTR
1	pTa	G1	-	+
2	pTa	G1	-	+
3	pTa	G2	-	-
4	pT1	G3	+	+
5	pTa	G2	-	+
6	pT3	G3	-	+
7	pT2	G3	-	+
8	pT1	G2	-	-
9	pT2	G3	-	+
10	pTa	G2	+	+
11	pT1	G2	-	1
12	pTa	G2	-	+
13	pT1	G3	-	-
14	pTa	G2	-	-
15	pT1	G3	-	+
16	pT1	G3	-	+
17	pT2	G3	-	+
18	pTa	G2	-	-
19	pT1	G2	-	+
20	pT1	G3	-	+
21	pT2	G3	-	+
22	pT2	G3	-	+
23	pTa	G2	-	+
24	pTa	G2	-	+
25	pT2	G3	-	+
26	pT3	G3	-	+
27	pTa	G1	-	+
28	pTa	G2	-	+
29	pT2	G2	-	+
30	pT2	G3	-	+

Table 2 shows a compilation of the results of the detection of hTR in the urine of patients with bladder carcinoma, benign urological diseases and healthy persons.

Table 2

Sample	**hTR-positive sample**	**hTR-negative samples**	**Total**	**Statistics**
urine from bladder carcinoma	25 (83 %)	5	30	
urine from benign diseases	4 (27 %)	11	15	
normal urine	3 (15 %)	17	20	
sensitivity				83 %
positive predictive value				81 %

Table 3 shows a comparison of the results from publications of three different authors who determined the telomerase activity in tissue samples, bladder rinses and the urine from patients with urinary bladder carcinomas.

Table 3

Sample	Müller et al.	Yoshida et al.	Kinoshita et al.
tissue	96 %	86 %	98 %
bladder rinse	73 %	-	84 %
urine	0 %	62 %	55 %

A comparison of table 2 and table 3 shows that the

method according to the invention is significantly superior to other non-invasive methods of the prior art.

Further results which were obtained with the hTR test are shown in the following tables 4 and 5. Table 4 shows results of the detection of hTR in patients with confirmed urinary bladder carcinoma in tissue samples, bladder rinses and urine samples.

Table 5 shows results for the detection of hTR in urine samples of patients with confirmed urinary bladder carcinoma, benign urological diseases (urolithiasis, urinary tract infections) and healthy volunteers.

Table 4

Sample	hTR-positive samples	hTR-negative samples	Total
tissue	27 (96 %)	1	28
bladder rinse	25 (89 %)	3	28
urine	23 (82 %)	5	28

Table 5

Sample	hTR-positive samples	hTR-negative samples	Total
urine of urinary bladder carcinoma	36 (80 %)	9	45
urine of benign diseases	2 (22 %)	7	9
normal urine	2 (7 %)	26	28

These results also confirm the high sensitivity and the high positive predictive value of the method according

to the invention.

Example 2

After adding 10^1 to 10^6 telomerase-positive J82 cells to 50 ml urine, the cells were isolated as described in example 1 and examined for the presence of the human telomerase RNA component by means of RT-PCR. Unequivocal positive results could be obtained with 10^4 and more cells per 50 ml urine.

In a parallel test the spiked urine samples were stored at 4°C and subsequently processed as described in example 1 and examined for the presence of the human telomerase RNA component by means of RT-PCR. After 48 h storage unequivocal positive results could also be obtained in this case with 10^4 and more cells per 50 ml urine.

After three days storage at 4°C or room temperature and subsequent processing as described above, the telomerase RNA component could still be unequivocally detected in urine samples of bladder carcinoma patients.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: DETECTION OF URINARY BLADDER CARCINOMA IN A URINE SAMPLE

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACTGAGAAG GGCGTAGGCG CCGTGC
26

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GTTTGCTCTA GAATGAACGG TGGAAG
26

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTCACCCCCA CTGAAAAAGA TGA
23

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCATCTTCA AACCTCCATG ATG 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AATCCGTCGA GCAGAGTT 18

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCCTTACCCT TACCCTTACC CTTA 24

Claims

1. Method for the detection of urinary bladder carcinoma in a urine sample,
wherein
(a) a urine sample is prepared and
(b) telomerase RNA is determined in the sample

2. Method as claimed in claim 1,
wherein
step (a) comprises isolating cells from the urine sample and lysing them.

3. Method as claimed in claim 1 or 2,
wherein
the telomerase RNA is determined by means of non-radioactive marker groups.

4. Method as claimed in one of the previous claims,
wherein
the determination of the telomerase RNA comprises a nucleic acid amplification step.

5. Method as claimed in one of the previous claims,
wherein
the determination of the telomerase RNA comprises a reverse transcription step.

6. Method as claimed in one of the claims 4 to 5,
wherein
a primer pair is used for the nucleic acid

amplification in the determination of a human telomerase RNA which is selected from:

(a) nucleotides with the nucleotide sequences shown in SEQ ID NO. 1 and SEQ ID NO.2 and

(b) nucleotides which contain a partial sequence of the nucleotide sequences shown in SEQ ID NO.1 and SEQ ID NO.2 having a length of at least 5 nucleotides.

7. Method as claimed in one of the claims 4 to 6, **wherein** the amplification product is analysed without separating the reaction mixture.

8. Method as claimed in claim 7, **wherein** the amplification product is immobilized on a solid phase.

9. Method as claimed in claim 7, **wherein** the amplification product is determined by on-line detection in a homogeneous test format.

10. Method as claimed in one of the previous claims, **wherein** in addition to the telomerase RNA in the sample, a standard nucleic acid that differs therefrom is determined.

11. Method as claimed in claim 10, **wherein** a primer pair is used for the determination of β2-microglobulin RNA as a human standard RNA which is

selected from:

(a) nucleotides with the nucleotide sequences shown in SEQ ID NO. 3 and SEQ ID NO. 4 and

(b) nucleotides which contain a partial sequence of the nucleotide sequences shown in SEQ ID NO.3 and SEQ ID NO.4 with a length of at least 5 nucleotides.

12. Method as claimed in one of the previous claims, **wherein**
the determination is carried out as a one-pot reaction.

13. Method as claimed in one of the previous claims, **wherein**
the telomerase RNA and optionally the standard nucleic acid are detected under conditions that allow a specific detection of the telomerase RNA and - if present - the standard nucleic acid without separation of amplification by-products.

14. Reagent kit for the detection of telomerase RNA comprising:

(a) a primer for the reverse transcription of a telomerase RNA

(b) an agent for reverse transcription,

(c) a primer pair for the amplification of a DNA obtained from telomerase RNA by reverse transcription,

(d) an agent for nucleic acid amplification and

(e) marker groups.

15. Use of the reagent kit as claimed in claim 14 in a non-invasive method for the detection of urinary bladder carcinoma.

16. Use as claimed in claim 15 in a method as claimed in one of the claims 1 to 14.