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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSING AND TREATING UROTHELIAL CANCER

(57) Abstract: A method of diagnosing urothelial cancer in a subject is provided. The method comprising: (a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein the presence and/or the level of the expression product of the H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and (b) wherein when an absence and/or a level of the expression product of the H19 gene below a predetermined threshold is indicated classifying the subject as free of the urothelial cancer without subjecting the subject to cystoscopy; and wherein when the presence and/or the level of the expression product of the H19 gene above a predetermined threshold is indicated corroborating presence of a cell of the urothelial cancer using the cystoscopy.

METHODS AND COMPOSITIONS FOR DIAGNOSING AND TREATING UROTHELIAL CANCER

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods and compositions for diagnosing and treating urothelial cancer.

Bladder cancer is the fourth most common malignancy in men and tenth in women worldwide. 90% of bladder cancer cases are urothelial carcinoma (UC), of which: 75% are papillary, not invasive at detection, but carrying high recurrence rate of about 50% - 70%; additional 20% are found to be more aggressive, penetrating the basement membrane or muscle-invasive, and the remaining 5% of the cases are metastatic at presentation [1, 2]. Currently, diagnosis and follow-up after initial trans-urethral resection of bladder cancer requires cystoscopy and urine cytology. These methods are endowed with many disadvantages. Cystoscopy is not 100% sensitive particularly for carcinoma in situ, it is invasive, unpleasant, expensive operator dependent, associated with morbidity, and patient compliance with surveillance recommendations is low [3]. Urine cytology is considered specific but less sensitive in low grade tumours [4, 5]. Although molecular profiling of tumours yielded useful biomarkers in several cancers such as breast cancer and melanoma, today no biomarker is used routinely in the clinical management of patients with bladder cancer [6].

The H19 gene is an imprinted gene located on chromosome 11, normally transcribed solely from the maternal allele [7, 8]. The H19 gene does not encode for a protein, its product is a long non-coding RNA which is expressed in the healthy foetus, drastically downregulated postnatally and is re-expressed in tumours arising from tissues which express it in foetal life [9]; therefore defined as an oncofetal RNA. High expression of H19 can be found in several tumors such as adrenal, pancreatic, bladder, ovarian and breast tumours, choriocarcinoma and glioblastoma (reviewed in [10]). Previous reports have suggested H19 as an oncogene which holds a significant role in the development and progression of tumors and metastatic spread [11-13]. The loss of the paternal imprinting augments H19 expression and was shown to be associated with neoplasia such as Wilm's tumour [14]. In addition, the H19 gene is proximal to *IGF2*

which is involved in cell cycle and differentiation; they both compete for common regulatory sequences and are reciprocally imprinted [15].

85 % of UC cells express high levels of the H19 gene [16], compared to healthy cells. H19 RNA was shown to enable cancer cells to proliferate under serum starved conditions *in vitro* [17] and to enhance tumour growth and angiogenesis of bladder cancer cells in vivo [11]. These results have led to evaluation of the use of *Diphtheria* toxin under the regulatory elements of H19 in clinical trials for bladder cancer therapy [16, 18, 19].

Additional Related Background Art:

I. Matouk et al. Cancer Therapy (2005) 3: 249-266;

US Patent No. US5955273; and

US Patent Application Publication No. US20060121477.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of diagnosing urothelial cancer in a subject, the method comprising:

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein the presence and/or the level of the expression product of the H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and

(b) wherein when an absence and/or a level of the expression product of the H19 gene below a predetermined threshold is indicated classifying the subject as free of the urothelial cancer without subjecting the subject to cystoscopy;

and wherein when the presence and/or the level of the expression product of the H19 gene above a predetermined threshold is indicated corroborating presence of a cell of the urothelial cancer using the cystoscopy.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring disease state in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein the presence and/or the level of the expression product of the H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and

(b) repeating the determining following a predetermined time interval, thereby monitoring the disease state in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of monitoring efficacy of cancer therapy in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject following the cancer therapy, wherein an absence of the expression product of the H19 gene and/or a decrease from a predetermined threshold in the level of the expression product of the H19 gene following the cancer therapy is indicative of reduction of cancerous cells, and efficaciousness of the cancer therapy.

According to an aspect of some embodiments of the present invention there is provided a method of predicting an efficacy of a cancer therapy for treatment of a subject diagnosed with urothelial cancer, the method comprising:

(a) contacting a biological fluid sample of a subject with a therapeutically effective amount of the cancer therapy; and

(b) determining a presence and/or a level of an expression product of a H19 gene in the biological fluid sample of the subject following the contacting with the cancer therapy;

wherein an absence and/or a decreased level of expression of the expression product of the H19 gene following the contacting with the cancer therapy relative to a level of expression of the expression product of the H19 gene prior to the contacting with the cancer therapy indicates that the cancer therapy is efficient for treating the subject, thereby predicting efficacy of the cancer therapy.

According to some embodiments of the invention, there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject according to the method; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) treating the subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

According to some embodiments of the invention, there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject according to the method; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) selecting a cancer therapy based on the presence and/or the level of the expression product of the H19 gene, thereby treating the subject diagnosed with urothelial cancer.

According to some embodiments of the invention, there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) treating the subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

According to some embodiments of the invention, there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) selecting a cancer therapy based on the level of the expression product of the H19 gene, thereby treating the subject diagnosed with urothelial cancer.

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) treating the subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

According to an aspect of some embodiments of the present invention there is provided a method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) selecting a cancer therapy based on the level of the expression product of the H19 gene, thereby treating the subject diagnosed with urothelial cancer.

According to some embodiments of the invention, the method further comprising repeating steps (a) and (b) following a predetermined time interval.

According to some embodiments of the invention, the method further comprising repeating the determining following a predetermined time interval.

According to some embodiments of the invention, the predetermined time interval comprises between one month and 6 months.

According to some embodiments of the invention, the repeating the determining comprises at least 3 tests.

According to some embodiments of the invention, the predetermined threshold is at least 150 copies per ml of the biological fluid sample.

According to some embodiments of the invention, the predetermined level is at least 150 copies per ml of the biological fluid sample.

According to some embodiments of the invention, the predetermined threshold is at least 950 copies per ml of the biological fluid sample.

According to some embodiments of the invention, the predetermined level is at least 950 copies per ml of the biological fluid sample.

According to some embodiments of the invention, the method comprising obtaining the biological fluid sample prior to the determining.

According to some embodiments of the invention, the presence and/or the level of H19 is determined in a cell comprised in the biological fluid sample.

According to some embodiments of the invention, the determining is effected by contacting the biological fluid sample with an agent capable of detecting H19 RNA or cDNA prior to the determining.

According to some embodiments of the invention, the method comprising separating a cell from the biological fluid sample prior to the determining.

According to some embodiments of the invention, the separating is effected by centrifugation.

According to some embodiments of the invention, the determining is effected by contacting the cell with an agent capable of detecting H19 RNA or cDNA prior to the determining.

According to some embodiments of the invention, the method comprising corroborating the presence of a cell of the urothelial cancer using a state of the art technique.

According to some embodiments of the invention, the state of the art technique is selected from the group consisting of urine cytology, cystoscopy and biopsy.

According to some embodiments of the invention, the contacting is effected *ex-vivo* or *in-vitro*.

According to some embodiments of the invention, the determining is effected by a method selected from the group consisting of PCR, RT-PCR and hybridization with a probe comprising a detectable moiety.

According to some embodiments of the invention, the biological fluid sample is selected from the group consisting of urine, blood, serum, lymph fluid, feces and rinse fluid that was in contact with the tumor.

According to some embodiments of the invention, the biological fluid sample is urine.

According to an aspect of some embodiments of the present invention there is provided a composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with highly recurring urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

According to an aspect of some embodiments of the present invention there is provided a composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with second recurrence of urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

According to an aspect of some embodiments of the present invention there is provided a composition of matter comprising RNA or cDNA from a urine sample of a

subject diagnosed with low grade urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

According to some embodiments of the invention, the agent capable of detecting H19 RNA or cDNA is an oligonucleotide which specifically hybridizes with RNA or cDNA of H19.

According to some embodiments of the invention, the urothelial cancer is selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer.

According to some embodiments of the invention, the highly recurring comprises a recurrence in a time period not exceeding 2 years.

According to some embodiments of the invention, the subject has not been subjected to an anti urothelial cancer therapy.

According to some embodiments of the invention, the subject has been subjected to an anti urothelial cancer therapy.

According to some embodiments of the invention, the subject has not been subjected to a surgery to remove an urothelial cancer.

According to some embodiments of the invention, the cancer therapy comprises a surgery.

According to some embodiments of the invention, the cancer therapy comprises a therapy selected from the group consisting of radiation therapy, chemotherapy and immunotherapy.

According to some embodiments of the invention, the cancer therapy comprises a DNA-based drug.

According to some embodiments of the invention, the DNA-based drug is based on the transcriptional regulatory elements of H19.

According to some embodiments of the invention, the DNA-based drug comprises an expression vector encoding a toxin e.g., *Diphtheria* Toxin A under the control of the H19 promoter e.g. BC-819.

According to some embodiments of the invention, the cancer therapy comprises an agent which downregulates an expression of H19.

According to some embodiments of the invention, the urothelial cancer is a bladder cancer.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

Figure 1 is a graph showing *H19* copy numbers per ml urine in healthy volunteers and patients with urothelial carcinoma of the bladder. Total RNA was extracted from cells pelleted from 30-50 ml fresh urine. Up to 50 ng RNA were used for reverse transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed in duplicates as described in the Methods, alongside negative and positive (plasmid-generated standard curve) controls. For the purpose of the presented plots (logarithmic scale), specimens yielding zero copies (either healthy or patients) were omitted. All specimens included, the min, 25^{th}, 50^{th}, 75^{th} percentiles and max values were [0, 0, 3, 67 and 23139 copies/ml] in healthy volunteers and [0, 721, 2748, 867311 and 42385704 copies/ml] in patients, respectively.

Figures 2A-B are Receiver Operating Characteristic (ROC) curves depicting the performance of urine *H19* copy numbers in classification of study participants as the discrimination threshold is varied. Areas under the curves are 0.886 in Figure 2A (*H19* copies per ng RNA, $p<0.0001$) and 0.933 in Figure 2B (*H19* copies per ml urine, $p<0.0001$).;

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods and compositions for diagnosing and treating urothelial cancer.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Contemporary diagnosis of urothelial cancer (UC) and follow-up of the patients is performed by invasive means and frequent insensitive cytology testing. H19 is a critical gene in cancer development and therefore it is a reliable biomarker for the detection of UC and its recurrence.

Whilst reducing the present invention to practice, the present inventors have found that the sensitivity of H19 expression level determination is superior to non-invasive diagnostic tools available today and therefore H19 determination is reliable enough to prevent unnecessary cystoscopies which cause tremendous suffer to the patients.

The present inventors have also found that H19 biomarker is a biomarker of UC patient subgroups and therefore can be used for determining disease course and personalized therapy. Hence the present inventors provide, for the first time, procedures and guidelines for avoiding cytoscopic evaluations, or reducing their frequency significantly.

Thus, according to an aspect of the invention there is provided a method of diagnosing urothelial cancer in a subject, the method comprising:

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein said presence and/or said level of said expression product of said H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and

(b) wherein when an absence and/or a level of said expression product of said H19 gene below a predetermined threshold is indicated classifying the subject as free of said urothelial cancer without subjecting the subject to cystoscopy;

and wherein when said presence and/or said level of said expression product of said H19 gene above a predetermined threshold is indicated corroborating presence of a cell of the urothelial cancer using said cystoscopy.

Unless otherwise defined herein, all definitions and descriptions are relevant to all aspects described herein.

As used herein the term "urothelial cancer" or "UC" or "bladder cancer" or "*transitional cell carcinoma* (TCC)", refers to the most common type of bladder cancer. This type of cancer typically starts in the urothelial cells that line the inside of the bladder. Urothelial cells also line other parts of the urinary tract, such as the part of the kidney that connects to the ureter (called the *renal pelvis*), the ureters, and the urethra. UC is often described based on invasiveness. Non-invasive cancers are still in the inner layer of cells (the transitional epithelium) but have not grown into the deeper layers. Invasive cancers have grown into deeper layers of the bladder wall. UC can also be classified as *superficial* or *non-muscle invasive*. These terms include both non-invasive tumors as well as any invasive tumors that have not grown into the main muscle layer of the bladder.

UCs are also divided into 2 subtypes, papillary and flat. Papillary carcinomas grow in slender, finger-like projections from the inner surface of the bladder toward the hollow center. Papillary tumors often grow toward the center of the bladder without growing into the deeper bladder layers. These tumors are called *noninvasive papillary cancers*. Very low-grade (slow growing), non-invasive papillary cancer is sometimes called *papillary urothelial neoplasm of low-malignant potential* (PUNLMP) and tends to have a very good outcome. Flat carcinomas do not grow toward the hollow part of the bladder at all. If a flat tumor is only in the inner layer of bladder cells, it is known as a *non-invasive flat carcinoma* or a *flat carcinoma in situ (CIS)*. If either a papillary or flat tumor grows into deeper layers of the bladder, it is called an *invasive urothelial (or transitional cell) carcinoma*.

According to the American Joint Committee on Cancer/International Union Against Cancer/Union Internationale Contre le Cancer (AJCC/UICC) TNM system[20], UC is staged as follows:

Tumor is characterized at its primary niche (T, wherein Tx stands for inability to assess primary tumor;T0-negation of tumor evidence; Ta-noninvasive papillary

carcinoma;Tis-carcinoma in situ;T1-tumor invades sub-epithelial connective tissue;T2-muscle invasion;T3-invasion to perivesical tissues; T4a-invasion to either prostate stroma, seminal vesicles, uterus or vagina, and T4b-metastasis to pelvic wall or abdominal wall) and may also have metastases in regional lymph nodes (N, wherein Nx stands for inability to assess nodes;N0-negation of regional node metastases;N1 & N2-one to multiple metastatic nodes, respectively and N3- metastasis in the common iliac lymph nodes) and in distant location (M1, as opposed to M0-no distant metastases). Based on the elaborated above TMN system, the overall staging definition may range from stages 0a and 0is (Ta and Tis, respectively, N0 and M0) via stages I to III(T1 to T4a, N0 and M0), to stage IV(with three sub-stages: T4b,N0,M0;any T,N1-3,M0;any T or N,M1).

The "subject" according to this aspect of the invention relates to a human subject who has never been diagnosed with UC or is on remission for at least 5 years but may exhibit symptoms of UC or is at risk of being affected by UC.

According to a specific embodiment, the subject has never been diagnosed with UC.

According to a specific embodiment, the subject has not been subjected to anti UC therapy.

The subject may be of any age, gender and ethnic group with the following notes. UC typically occurs in older people. About 9 out of 10 people with this cancer are over the age of 55. The average age at the time of diagnosis is 73. Men are about 3 to 4 times more likely to get UC during their lifetime than women. White Caucasians are diagnosed with UC about twice as often as African Americans or Hispanic Americans.

Signs and symptoms of UC include blood in the urine and changes in urination, as further explained hereinbelow.

Blood in the urine - In most cases, blood in the urine (*hematuria*) is the first sign of UC. Usually, the early stages of UC cause bleeding but little or no pain or other symptoms.

Changes in bladder habits or symptoms of irritation - UC can sometimes cause changes in urination, such as: having to urinate more often than usual; pain or burning sensation during urination.

Risks for UC include, but are not limited to, smoking, exposure to aromatic amines, use of medicines and herbal supplements of specific groups e.g., pioglitazone (Actos), arsenic in drinking water, not drinking enough fluids, race and ethnicity, age, gender, chronic bladder infections and bladder birth defects.

As used herein "diagnosis" or "diagnosing" refers to determining presence or absence of a pathology (e.g., a disease, disorder, condition or syndrome) in this case, UC, classifying a pathology or a symptom, determining a severity of the pathology, monitoring pathology progression, forecasting an outcome of a pathology and/or prospects of recovery and screening of a subject for a specific disease.

As used herein the phrase "expression product of H19" refers to a transcriptional product of the H19 gene (GenBank Accession No. M32053, or Gene ID: 283120 SEQ ID NO: 9).

As used herein "biological fluid sample" refers to a body fluid such as whole blood, serum, plasma, cerebrospinal fluid, urine, lymph fluids, and various external secretions as well as rinse fluids.

According to a specific embodiment, the sample comprises cells.

According to a specific embodiment, the sample does not comprise cells (e.g., plasma).

According to a specific embodiment, the biological sample comprises urine or blood.

According to a specific embodiment, the presence and/or level of H19 expression product is determined in a cell comprised in the biological sample. According to a specific embodiment, the urine sample or blood sample is processed to obtain the cell fraction e.g., by centrifugation. In the case of urine, the urine sample is processed to obtain urine sediment cells. Thus, according to a specific embodiment, urinary cells are thus pelleted from the specimens and total RNA is extracted, quantified as further described hereinbelow e.g., reverse-transcribed by random priming.

According to a specific embodiment, the biological sample is obtained or retrieved from the subject prior to the molecular determination of the RNA levels/presence.

Once RNA is at hand, the presence of H19 RNA or level may be detected by methods known in the art such as PCR, RT-PCR, in situ PCR, in situ RT-PCR, LCR and, 3SR, and hybridization with a probe comprising a detectable moiety.

These methods typically make use of agents that are capable of detecting H19 RNA or cDNA (e.g., oligonucleotide primers/probes).

According to a specific embodiment, the level of the H19 expression product is determined by quantitative PCR (qPCR).

Non-limiting examples of primers and probes that can be used to determine the presence and/or level of H19 expression product are provided in Table 2 hereinbelow.

According to a specific embodiment, the determination is effected using an agent capable of detecting H19 RNA or cDNA such as DNA binding dyes or fluorescent probes such as those typically used in real-time PCR (qPCR).

According to some embodiments of the present invention, a predetermined threshold, is at least 150 copies per ml of the biological sample (e.g., as determined by qPCR on sedimented urine cell sample).

As stated above, the present teachings are particularly critical for avoiding unnecessary cytoscopic examinations which cause the subject significant suffering.

Hence, the method according to this aspect, i.e., steps (a) and (b) are repeated following a predetermined time interval. The physician e.g., oncologist/urologist/internal medicine may inform the client of the results and provided these are below the predetermined threshold subscribe another test following e.g., 1-6 months, 3-6 months, 4-6 months, 2-4 months.

Accordingly the test may be repeated 2 times, 3 times or more e.g., at least 3 times.

As used herein "without subjecting the subject to cytoscopy" means 2-6 months after diagnosis is made.

According to another aspect there is provided a method of monitoring disease state in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein said presence and/or said level

of said expression product of said H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer (as described above); and

(b) repeating said determining following a predetermined time interval (as described above), thereby monitoring the disease state in the subject.

As used herein "low grade UC" refers to non-muscle invasive tumors at stages below T1, including PUNLMP(see above) and excluding Tis.

As used herein "second recurrence of UC" refers to repeated occurrence of urothelial tumor after initial alleged successful response to treatment e.g., intravesical treatment.

As used herein "highly recurring UC" refers to more than one repeated occurrence of urothelial tumor after previous alleged successful response to treatment e.g., intravesical treatment.

Monitoring of UC can be further corroborated using methods which are well known in the art. Thus, embodiments of this aspect of the invention relate to corroborating the presence or the cell of the UC using a state of the art technique. These include, but are not limited to, medical history and physical examination, Imaging (MRI, ultrasonography, intravenous urography (IVU), computed tomography(CT))), urine lab tests (urinalysis, urine cytology, urine culture) urine tumor marker tests (e.g., BTA, NMP22, UroVysion) or cytoscopy.

According to another aspect of the invention there is provided a method of monitoring efficacy of cancer therapy in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject following the cancer therapy, wherein an absence of said expression product of said H19 gene and/or a decrease from a predetermined threshold in the level of said expression product of said H19 gene following the cancer therapy is indicative of reduction of cancerous cells, and efficaciousness of the cancer therapy.

According to another aspect of the invention, there is provided a method of predicting an efficacy of a cancer therapy for treatment of a subject diagnosed with urothelial cancer, the method comprising:

(a) contacting a biological fluid sample of a subject with a therapeutically effective amount of the cancer therapy; and

(b) determining a presence and/or a level of an expression product of a H19 gene in said biological fluid sample of said subject following said contacting with said cancer therapy;

wherein an absence and/or a decreased level of expression of said expression product of said H19 gene following said contacting with said cancer therapy relative to a level of expression of said expression product of said H19 gene prior to said contacting with said cancer therapy indicates that the cancer therapy is efficient for treating the subject, thereby predicting efficacy of the cancer therapy.

As used herein "presence" or "absence" is determined by a PCR/hybridization based method e.g., qPCR.

As used "decreased " refers to at least 10 % reduction, at least 20 % reduction, at least 30 % reduction, at least 40 % reduction, at least 50 % reduction, at least 60 % reduction, at least 70 % reduction, at least 80 % reduction, at least 90 % reduction, at least 95 % reduction in expression product of the H19 gene (e.g., as determined by qPCR).

According to a specific embodiment, decreased is at least 40 % reduction in expression product of the H19 gene (e.g., as determined by qPCR).

As used herein the sample refers to a cellular biological sample (e.g., as described above).

Contacting is typically effected ex-vivo.

Examples of such therapies include, but are not limited to, intravesical therapy chemotherapy, immunotherapy and a DNA-based drug (e.g., such as based on the transcriptional regulatory elements of H19).

According to yet another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject as described above; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

Treatment of bladder cancer is based on the tumor's clinical stage, which is how deep it is thought to have grown into the bladder wall and whether it has spread beyond the bladder. Other factors, such as the size and grade of the tumor and a person's overall health, can also affect treatment options. The skilled artisan is aware of the treatment options and the significance of predicting the outcome of treatment.

When needed, according to the present teachings, treating of UC can be effected using methods which are well known in the art. These include, but are not limited to, surgery (TURBT, cystectomy), intravesical therapy (e.g., BCG), chemotherapy (e.g. Mitomycin C), radiation therapy and immunotherapy. Treatment can include each of these options or combinations thereof (e.g., TURBT, BCG).

According to a specific embodiment, treatment is effected using a DNA-based drug.

According to a specific embodiment, said DNA-based drug is based on the transcriptional regulatory elements of H19.

According to a specific embodiment, the DNA-based drug comprises an expression vector encoding a toxin e.g., *Diphtheria* Toxin A under the control of the H19 promoter e.g. BC-819.

For example Scaiewicz et al. J Oncol. 2010;2010:178174 teach nonviral vector, expressing the *Diphtheria* toxin A chain under the control of the H19 gene regulatory sequences and is hereby incorporated by reference in its entirety.

According to a specific embodiment, the cancer therapy comprises an agent which down regulates an expression or activity of H19.

These drugs are based on for example genome editing agents such as CRISPR/Cas9 as well as RNA silencing agents.

Examples of specific siRNAs which can effectively down-regulate H19 mRNA are disclosed in U.S. Pat. No. 8,067,573, which is hereby incorporated by reference in its entirety.

According to still another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject according to the method described hereinabove; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) selecting a cancer therapy based on the presence and/or the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer. The selection of the treatment regimen may depend on other factors as well (such as tumor size, other markers, stage etc.). The skilled artisan will know how to integrate all these parameters when selecting the treatment regimen.

According to still another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method described herein; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

According to still another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method described herein; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) selecting a cancer therapy based on the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer.

According to still another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

According to still another aspect of the invention there is provided a method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) selecting a cancer therapy based on the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer.

Also provided is a composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with second recurrence of urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

Yet provided is a composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with low grade urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

Yet provided is a composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with highly recurrent urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

The present teachings also relate to kits which comprise agents which can be used to determine the presence, absence, level of an H19 expression product specifically. Such kits typically include instructions for use as well as control reagents and may also include auxiliary reagents (such as general buffers, rinses etc.)

Thus, components of such kits may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more tests. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of diagnostics/theranostics, which notice is reflective of approval by the agency of the form of the compositions for human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is understood that any Sequence Identification Number (SEQ ID NO) disclosed in the instant application can refer to either a DNA sequence or a RNA sequence, depending on the context where that SEQ ID NO is mentioned, even if that SEQ ID NO is expressed only in a DNA sequence format or a RNA sequence format. For example, SEQ ID NO: is expressed in a DNA sequence format (*e.g.*, reciting T for thymine), but it can refer to either a DNA sequence, or the RNA sequence of an RNA molecule nucleic acid sequence. Similarly, though some sequences are expressed in a RNA sequence format (*e.g.*, reciting U for uracil), depending on the actual type of molecule being described, it can refer to either the sequence of a RNA molecule comprising a dsRNA, or the sequence of a DNA molecule that corresponds to the RNA sequence shown. In any event, both DNA and RNA molecules having the sequences disclosed with any substitutes are envisioned.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various

embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); “Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames,

B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Objective **-** The objective of this study was to evaluate *H19* RNA expression in urine sediment cells as a mean to diagnose UC.

Methods **–** Urine samples were collected from healthy volunteers (n=27), and from patients currently suffering from UC diagnosed with cystoscopy and confirmed by pathology (n=21). Urinary cells were pelleted from the specimens and total RNA was extracted, quantified and reverse-transcribed by random priming. The data was incorporated to receiver operating characteristic (ROC) curves and the sensitivity and specificity were calculated with regard to the presence of UC diagnosed by pathology.

Results **-** *H19* RNA was unequivocally detected in the urine of 19/21 patients (90.5 %) and in 7/27 healthy volunteers (25.9%). *H19* copy numbers were 3 orders of magnitude higher in patients' urine compared to controls (median 2750 versus 3 copies per ml, respectively). Using *H19* copies in each sample for ROC curve analysis, the maximal sensitivity of the test was 95.5 % and the maximal specificity 74.1 % (area under the ROC curve 0.933).

Conclusions **-** Urinary cell *H19* transcript level determination is a highly sensitive test for detection of UC and with further studies can be transformative technique for diagnosing and following UC patients.

EXAMPLE 2

Material and Methods

Subjects - Two groups of volunteers were recruited to this case control study: (1) patients presenting to the urology outpatient clinic or inpatient service with a bladder tumour, and (2) healthy volunteers without known urinary tract disease. Patients were excluded if diagnosis of urothelial carcinoma was not confirmed by pathological examination of the biopsy or the excised tumour. The study was approved by the Helsinki committee of the Hadassah – Hebrew University Medical Centre.

Clinical characteristics of study participants - The research group included 58 subjects; 27 healthy volunteers and 29 patients presenting to the urology service (Table 1 below). We excluded eight patients; seven cystoscopy did not demonstrate visible tumour, or the pathology results were not supportive of tumour presence. One patient was not operated by the time the samples were processed and analysed (thus pathological confirmation is lacking). Healthy volunteers were 37±7 years old and patients' age was 69±12 years. In all but one patient, tumour stage was T1/Ta. Three of the 21 patients (14 %) had high-grade tumours.

Clinical specimens - Urine specimens were obtained from study participants following written informed consent. Volunteers provided a single fresh urine void in sterile 100 ml containers. Containers were placed in an ice bucket and transferred to the laboratory within 1 hour. Up to 50 ml of urine were transferred to 50 ml conical (Falcon) tubes and spun at 2,000 RPM, at 4°C for 5 min. Stick urinalysis was performed on remaining urine. Following centrifugation, the urine supernatant (all except ~0.5 ml) was transferred to a new Falcon tube (stored for possible future study) while 1 ml PBS was added to the pellet and the remaining ~ 0.5 ml of urine. The cell pellet was resuspended by pipetting and transferred to a siliconised 1.5 ml tube (Sigma). 10-20 µl were pipetted onto a microscope glass slide (and the presence of cells was recorded).The remaining was spun at 2,000 g (Neofuge 13R, Heal Force), 4°C, 5 min. All but ~100 µl of the supernatant was discarded, and the remaining sediment was flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction, quantification and reverse transcription - Upon completion of subject recruitment, sample collection and processing, 300 µl of TRIzol LS were added to each cell pellet. Cells were lysed and the solution homogenised by 1 min vortexing

and the tubes were incubated 5 min at room temperature. Chloroform (80 µl) was added followed by 30 sec vortexing and 15 min centrifugation at 12,000 g, 4°C. The aqueous phase was transferred to a new siliconised tube on ice and 15 µg glycogen were added and briefly vortexed. One volume of isopropanol was added, mixed by flipping, and the tube stored at -20°C overnight. The mixture was spun at 21,000 g, 4°C, 30 min. The supernatant was discarded and the remaining fluid was re-spun briefly and pipetted out, followed by air-drying of the pellet for 5 min. The pellets were resuspended in 15 µl water (Synergy Ultrapure Water Purification System, EMD Millipore) and incubated 5 min at 60°C. Two µl were used for measurement of RNA concentration using the Qubit fluorometer (RNA HS Assay Kit, Life Technologies). Up to 50 ng RNA were used for reverse transcription, which was primed with random hexamers (High-Capacity cDNA Reverse Transcription Kit, Life Technologies). The cDNA was diluted (2.5-fold) to 50 µl using water.

RNA yield in urine sediment cells - Total RNA was extracted from urine sediment cells (20-50 ml fresh urine). RNA quantity (Table 1 below) was higher in sediment cells from patients (314 ng, IQR 132-515) as compared to healthy volunteers (22 ng, IQR 7-52) due to the presence of a higher number of cells and likely also due to larger amounts of extracellular RNA carried along with the cell pellets in patients' specimens.

Quantitative polymerase chain reaction and standard curve analysis - Quantitative PCR was carried out in duplicates on the 7900HT Fast Real-Time PCR System (Applied Biosystems) with a 96-well block. Each 10 µl assay consisted of 5 µl reaction mix (TaqMan Fast Universal PCR Master Mix), 0.5 µl of a 20:1 primer/probe mixture, 1 µl cDNA template or standard (PCR amplicon) and 3.5 µl water. The forward and reverse primers and probes used for *H19* or *ACTB* are listed in Table 2 below. Reaction steps were 20 sec 95°C followed by 40 cycles of 1 sec 95°C and 20 sec 60°C. Results were collected on both absolute quantities using the standard curve (see below) and categorical detection according to the device software-generated warnings (flags). Flags denoting absence of amplification in both duplicate wells were interpreted as negative results, while absence of these warnings in both duplicate wells was interpreted as positive results. Warning in 1 of 2 duplicate wells was interpreted as a borderline result. Absolute quantification was enabled by assaying a serially diluted

PCR amplicon in parallel to the study samples. The amplicon was generated by amplifying a section of the *H19* encompassing the qPCR assay region from HEK293 cDNA (forward primer CCCAGAACCCACAACATGAA/SEQ ID NO: 1; reverse primer AGTCGTGGAGGCTTTGAATC/SEQ ID NO: 2). The amplicon (426 bp) was extracted from EtBr-stained agarose gel, the DNA quantified using Qubit (dsDNA BR Assay Kits, Life Technologies), and copy number concentration was calculated.

Statistical analysis **-** Numerical data are presented as mean ± standard deviation (SD) or median and 25^{th} – 75^{th} percentile (IQR). Mann-Whitney U-tests were used to compare H19 copy numbers in patients versus healthy volunteers (R statistical platform). Box plots were generated using the ggplot2 package for R [21]. Receiver operating characteristic curves were generated and plotted using the pROC package for R [22].

Table 1: Study volunteer and specimen characteristics

	Healthy volunteers	UC patients
Male : female	25 : 2	18 : 3
Age, years	37 ± 7	69 ± 12
Total RNA in sediment cells, ng	22 (IQR 7-52)	314 (IQR 132-515)

UC, urothelial carcinoma (bladder); IQR, interquartile range

Table 2: List of primers and probes

Primer/probe	Sequence/SEQ ID NO:
H19 upstream	5'-TGCTGCACTTTACAACCACTG-3/3'
H19 downstream	5'-ATGGTGTCTTTGATGTTGGGC-3'/4
H19 probe	56-AM/TCCTCTAGC/ZEN/TTCACCTTCCAGAGCCGA/3IABkFQ/5
ACTB upstream	5'-GACAGGATGCAGAAGGAGATCACTG-3'/6
ACTB downstream	5'-CGCCGATCCACACGGAGTACTT-3'/7
ACTB probe	56-FAM/ATGAAGATC/ZEN/AAGATCATTGCTCCTCCT/3IABkFQ/8

Results

Detection of H19 RNA – categorical results

H19 was detected in 19 of the 21 patients (90 %) and was borderline-detected (namely, inconsistent results in duplicate qPCR wells; see Methods) in one additional patient (5 %). Both patients with false negative/borderline results had low-grade T1/Ta tumours. Among healthy volunteers, *H19* was detected in 7/27 patients (25.9 %) and

was borderline in two additional subjects (7.4 %). The performance of binary classification of subjects according to categorical detection of H19 was examined. Notably, sensitivity of 95.5 % was observed with specificity of 66.7 % when counting borderline results as positive, while if borderline results are counted as negative sensitivity is 90.9 % and specificity is 74.1 %. Simulations on negative and positive predictive values are provided in Table 3 below.

Quantitative detection of urine cell H19 expression - *H19* copy numbers, as determined through the use of an absolute standard curve, were 3 copies/ml urine in healthy volunteers (interquartile range (IQR) 0-67) and 2750 (IQR 720-867,310) copies/ml urine in patients (Figure 1). ROC curve analysis is presented in Figure 2 (area under the ROC curve is 0.933). Among our study volunteers, UC detection by urine cell *H19* quantification is 95.5 % sensitive and 88.9 % specific at a cut-off value of 156 copies per ml. At a cut-off value of 957 copies per ml, respective values are 72.7% and 96.3%.

Table 3: Performance of binary classification of subjects according to categorical detection of *H19* in urine sediment cells

	Sensitivity	Specificity	NPV prevalence ~ 20%†	PPV prevalence ~ 20%†	NPV prevalence ~ 5%†	PPV prevalence ~ 5%†
borderline counted positive*	95.5%	66.7%	98.3%	41.7%	99.6%	13.1%
borderline counted negative*	90.9%	74.1%	97.0%	46.7%	99.4%	15.6%

*, Negative status was defined as 2/2 duplicate PCR wells being labelled with "no amplification" flag (warning); positive status was defined as 0/2 duplicate wells flagged; and borderline status was defined as 1/2 PCR wells labelled with "no amplification" flag.
†, Negative (NPV) and positive (PPV) predictive value simulations assuming 20% or 5% prevalence of UC among subjects referred for the test.

Discussion

H19 is a gene of major importance in human cancer, involved in critical processes from primary tumorigenesis to metastasis [11, 13], thus it is an ideal marker for cancer

development. The comparison of *H19* expression levels in cells found in urine of patients with UC versus healthy volunteers exposes great differences. As a screening and follow up test, the most important parameter found in our study was the outstanding sensitivity of urine cell *H19* levels compared to published contemporary tests (Table 4 below). Specifically, in our study quantification of *H19* RNA afforded 91-96 % sensitivity, depending on cut-off definitions. Notably, we found high levels of *H19* gene expression in specimens from patients with predominantly low grade UC, a subtype which tends to recur in 50 % of cases and is often undetectable by cytology. It is worthwhile mentioning that two patients who demonstrated bladder tumours on cystoscopy and pathology had nearly undetectable levels of *H19*.

In this study, low-level *H19* expression was found in urine cells of seven control patients, without known UC. A possible explanation may be that *H19* which is essential for early tumor development is detected while current methods can't yet identify pre-cancerous cells. A technical reason may be that despite using a specific set of qPCR primers and probe, low-level non-specific amplification is still conceivable (due to mispriming).

The European Association of Urology guidelines from 2013 recommended that low risk patients be followed-up by cystoscopy and cytology 3 months post-surgery and then every 6 months for 5 years [1]. In high risk patients, cystoscopy and cytology will be performed in 3 months intervals for 2 years, then semi-annually for additional 5 years. In addition to above-mentioned limitations, cystoscopy carries a risk of causing urinary tract infection (UTI), sepsis, urethral strictures and perforation of the bladder. Prior to the test a urine culture is required and patients with asymptomatic bacteriuria require an antibiotic course. Cystoscopies are also expensive, cause great distress in some patients and sometimes are carried out in the operating theatre or in the clinic under sedation. Cytology examination requires an expert cytologist, which makes it operator dependent. In contrast, the use of RT-qPCR, for *H19* as a diagnostic means for UC detection is not operator dependent, caries no pain for the patient and is significantly more sensitive than the current available tests, giving the urologist a powerful tool for screening but more importantly for follow-up of patients with UC.

Table 4: Performance of the currently presented method followed by that of commercially and clinically available tests [23]

Test	Sensitivity	Specificity	Reference
H19, borderline counted positive	95.5%	66.7%	current study
H19, borderline counted negative	90.9%	74.1%	current study
Cytology	45.5%	89.5%	[4, 5]
BTA *stat*	52.5%	76.7%	[23]
NMP22	51%	96%	[24]
UroVysion	68.8%	89.1%	[25]
ImmunoCyt	78.3%	73.8%	[26]

H19 urine levels are age dependent

The present inventors tested whether the age of the healthy volunteers (being younger than the test group) was of significance to the study's results. The present inventors thus evaluated the relationship between *H19* urine levels and age. Pearson correlation between H19 copy numbers and age was -0.126 in the UC group (p=0.581) and -0.081 (p=0.783) in healthy volunteers, ruling out a significant association of urine *H19* with age.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

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26. Toma, M.I., et al., *Comparison of the ImmunoCyt test and urinary cytology with other urine tests in the detection and surveillance of bladder cancer.* World journal of urology, 2004. **22**(2): p. 145-9.

WHAT IS CLAIMED IS:

1. A method of diagnosing urothelial cancer in a subject, the method comprising:

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein said presence and/or said level of said expression product of said H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and

(b) wherein when an absence and/or a level of said expression product of said H19 gene below a predetermined threshold is indicated classifying the subject as free of said urothelial cancer without subjecting the subject to cystoscopy;

and wherein when said presence and/or said level of said expression product of said H19 gene above a predetermined threshold is indicated corroborating presence of a cell of the urothelial cancer using said cystoscopy.

2. A method of monitoring disease state in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising

(a) determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject, wherein said presence and/or said level of said expression product of said H19 gene above a predetermined threshold is indicative of presence of a cell of the urothelial cancer; and

(b) repeating said determining following a predetermined time interval,

thereby monitoring the disease state in the subject.

3. A method of monitoring efficacy of cancer therapy in a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising determining a presence and/or a level of an expression product of a H19 gene in a biological fluid sample of the subject following the cancer therapy, wherein an absence of said expression product of said H19 gene and/or a decrease from a

predetermined threshold in the level of said expression product of said H19 gene following the cancer therapy is indicative of reduction of cancerous cells, and efficaciousness of the cancer therapy.

4. A method of predicting an efficacy of a cancer therapy for treatment of a subject diagnosed with urothelial cancer, the method comprising:

(a) contacting a biological fluid sample of a subject with a therapeutically effective amount of the cancer therapy; and

(b) determining a presence and/or a level of an expression product of a H19 gene in said biological fluid sample of said subject following said contacting with said cancer therapy;

wherein an absence and/or a decreased level of expression of said expression product of said H19 gene following said contacting with said cancer therapy relative to a level of expression of said expression product of said H19 gene prior to said contacting with said cancer therapy indicates that the cancer therapy is efficient for treating the subject, thereby predicting efficacy of the cancer therapy.

5. A method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject according to the method of claim 1; and wherein when a predetermined level of expression product of H19 gene is indicated (b)

treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

6. A method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) diagnosing the subject according to the method of claim 1; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) selecting a cancer therapy based on the presence and/or the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer.

7. A method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method of claim 2; and wherein when a predetermined level of expression product of H19 gene is indicated (b) treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

8. A method of treating a subject diagnosed with urothelial cancer, the method comprising:

(a) monitoring the subject according to the method of claim 2; and wherein when a predetermined level of expression product of H19 gene is indicated

(b) selecting a cancer therapy based on the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer.

9. A method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) treating said subject with a cancer therapy, thereby treating the subject diagnosed with urothelial cancer.

10. A method of treating a subject diagnosed with urothelial cancer selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer, the method comprising:

(a) determining a level of an expression product of a H19 gene in a biological fluid sample of the subject; and wherein when a predetermined level of expression product of H19 gene is indicated;

(b) selecting a cancer therapy based on the level of said expression product of said H19 gene, thereby treating the subject diagnosed with urothelial cancer.

11. The method of claim 1, further comprising repeating steps (a) and (b) following a predetermined time interval.

12. The method of any one of claims 3, 9 and 10, further comprising repeating said determining following a predetermined time interval.

13. The method of any one of claims 2, 7, 8, 11 and 12, wherein said predetermined time interval comprises between one month and 6 months.

14. The method of any one of claims 2, 7, 8 and 11-13, wherein said repeating said determining comprises at least 3 tests.

15. The method of any one of claims 1, 2 and 5-8, wherein said predetermined threshold is at least 150 copies per ml of said biological fluid sample.

16. The method of any one of claims 5-10, wherein said predetermined level is at least 150 copies per ml of said biological fluid sample.

17. The method of any one of claims 1, 2 and 5-8, wherein said predetermined threshold is at least 950 copies per ml of said biological fluid sample.

18. The method of any one of claims 5-10, wherein said predetermined level is at least 950 copies per ml of said biological fluid sample.

19. The method of any one of claims 1-18, comprising obtaining said biological fluid sample prior to said determining.

20. The method of any one of claims 1-19, wherein said presence and/or said level of H19 is determined in a cell comprised in said biological fluid sample.

21. The method of any one of claims 1-20, wherein said determining is effected by contacting said biological fluid sample with an agent capable of detecting H19 RNA or cDNA prior to said determining.

22. The method of any one of claims 1-20, comprising separating a cell from said biological fluid sample prior to said determining.

23. The method of claim 22, wherein said separating is effected by centrifugation.

24. The method of any one of claims 22-23, wherein said determining is effected by contacting said cell with an agent capable of detecting H19 RNA or cDNA prior to said determining.

25. The method of claim 2, comprising corroborating said presence of a cell of the urothelial cancer using a state of the art technique.

26. The method of claim 25, wherein said state of the art technique is selected from the group consisting of urine cytology, cystoscopy and biopsy.

27. The method of claim 4, wherein said contacting is effected *ex-vivo* or *in-vitro*.

28. The method of any one of claims 1-27, wherein said determining is effected by a method selected from the group consisting of PCR, RT-PCR and hybridization with a probe comprising a detectable moiety.

29. The method of any one of claims 1-28, wherein said biological fluid sample is selected from the group consisting of urine, blood, serum, lymph fluid, feces and rinse fluid that was in contact with the tumor.

30. The method of any one of claims 1-28, wherein said biological fluid sample is urine.

31. A composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with highly recurring urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

32. A composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with second recurrence of urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

33. A composition of matter comprising RNA or cDNA from a urine sample of a subject diagnosed with low grade urothelial cancer, and an agent capable of detecting H19 RNA or cDNA.

34. The method of any one of claims 21 and 24 or the composition of matter of any one of claims 31-33, wherein said agent capable of detecting H19 RNA or cDNA is an oligonucleotide which specifically hybridizes with RNA or cDNA of H19.

35. The method of any one of claims 1 and 4-6, wherein said urothelial cancer is selected from the group consisting of low grade urothelial cancer, second recurrence of urothelial cancer and highly recurring urothelial cancer.

36. The method of any one of claims 2, 3, 7-10 and 35 or the composition of matter of claim 31, wherein said highly recurring comprises a recurrence in a time period not exceeding 2 years.

37. The method of claim 1, wherein said subject has not been subjected to an anti urothelial cancer therapy.

38. The method or the composition of matter of any one of claims 1-36, wherein said subject has been subjected to an anti urothelial cancer therapy.

39. The method or the composition of matter of any one of claims 1-38, wherein said subject has not been subjected to a surgery to remove an urothelial cancer.

40. The method or the composition of matter of any one of claims 3, 5-10 and 38, wherein said cancer therapy comprises a surgery.

41. The method or the composition of matter of any one of claims 3-10 and 38, wherein said cancer therapy comprises a therapy selected from the group consisting of radiation therapy, chemotherapy and immunotherapy.

42. The method or the composition of matter of any one of claims 3-10 and 38, wherein said cancer therapy comprises a DNA-based drug.

43. The method or the composition of claim 42, wherein said DNA-based drug is based on the transcriptional regulatory elements of H19.

44. The method or the composition of claim 42, wherein said DNA-based drug comprises an expression vector encoding a toxin e.g., *Diphtheria* Toxin A under the control of the H19 promoter e.g. BC-819.

45. The method or the composition of matter of any one of claims 3-10, 38, 42 and 43, wherein said cancer therapy comprises an agent which downregulates an expression of H19.

Figure 1

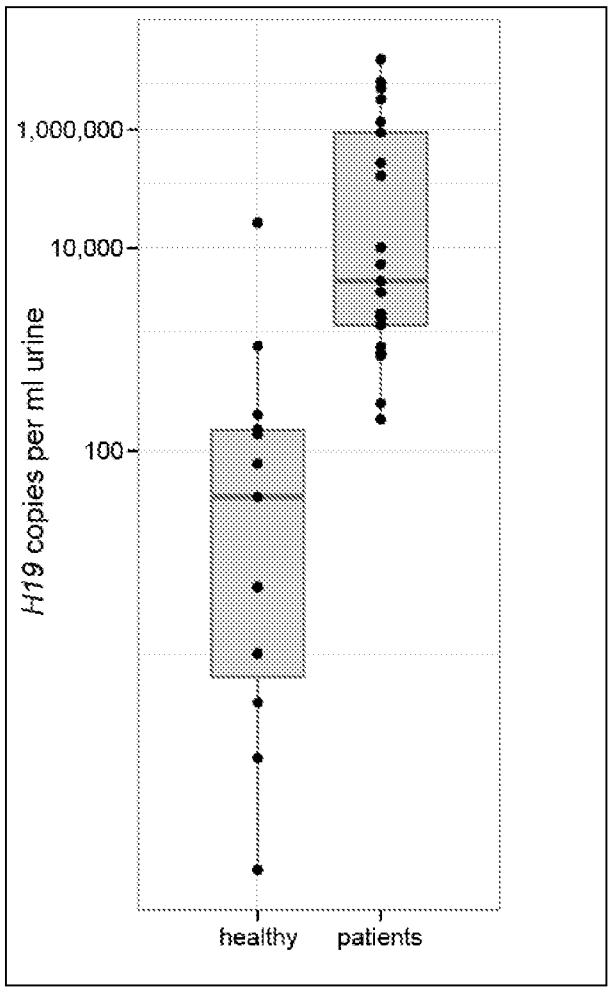
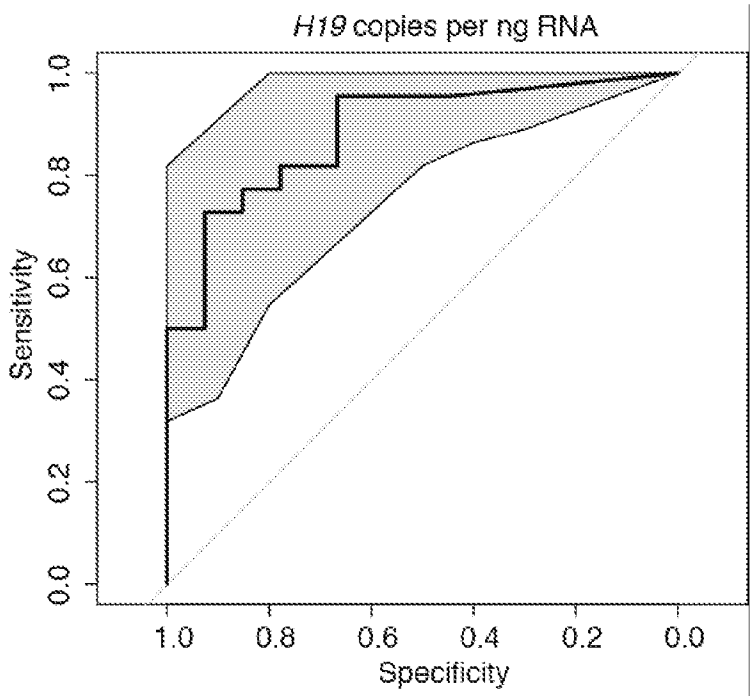


Figure 2A



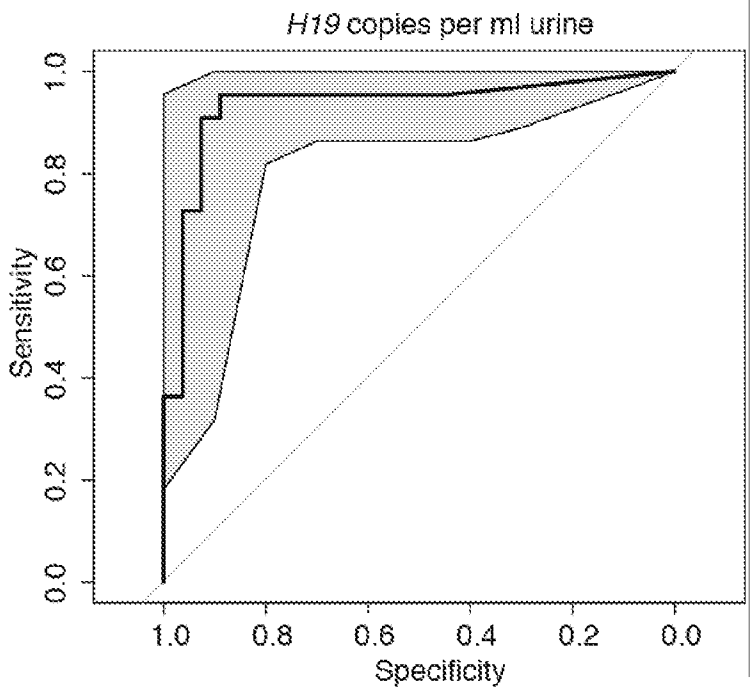


Figure 2B

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2016/050637

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUO MING ET AL: "Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression", CANCER LETTERS, NEW YORK, NY, US, vol. 333, no. 2, 24 January 2013 (2013-01-24), pages 213-221, XP028581872, ISSN: 0304-3835, DOI: 10.1016/J.CANLET.2013.01.033 the whole document	1-45
X	WO 2004/024957 A2 (YISSUM RES AND DEV [IL]; HOCHBERG ABRAHAM [IL]; AYESH SUHAIL [IL]; MCI) 25 March 2004 (2004-03-25)	31-33
Y	paragraph [0054]; claims 1,2,6	1-30, 34-45

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2016	07/11/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cornelis, Karen

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	H.-M. BYUN ET AL: "Examination of IGF2 and H19 Loss of Imprinting in Bladder Cancer", CANCER RESEARCH, vol. 67, no. 22, 15 November 2007 (2007-11-15), pages 10753-10758, XP055006135, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-07-0329 the whole document	1-45
Y	WO 95/24503 A1 (RAPAPORT ERICH [IL]; YISSUM RES DEV CO [IL]; HADASIT MED RES SERVICE [) 14 September 1995 (1995-09-14) the whole document	1-30, 34-45
A	WO 2007/007317 A1 (YISSUM RES DEV CO [IL]; HOCHBERG AVRAHAM [IL]; MATOUK IMAD [IL]) 18 January 2007 (2007-01-18) the whole document	1-45

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2016/050637

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