and those found in the ctDNA. Consequently, the ctDNA-based NGS analysis could revolutionize the management of patients suffering from cancer pathologies which are potentially curable or metastatic.

Document US 2013/143747 describes methods for a molecular classification of diseases and particularly molecular markers for cancer. The methods include analysis of panels of genes obtained from a blood sample. Synthesized mRNA or cDNA are obtained from the sample and the mutational status of the genes of the panels are determined. The presence of a particular mutational status in particular genes in a certain panel indicates that (a) the patient had a cancer and/or (b) the patient has that particular cancer. By the methods described in US 2013/143747 a screening is carried out with the purpose of finding people that already have a cancer, and even people that have a particular cancer. The patient has already developed the disease, even at an advanced step. US 2013/143747 does not provide that a genetic instability index can also be defined on the basis of an increase in the frequency of mutations with respect to one or more previous evaluation cycles Nor this document suggests that a predetermined set of genes and selected mutations can be defined in view of the subject's anamnesis. According to the methods of US 2013/143747, the type of genes in which mutations occur determine which tumor has been found, and an actual and real diagnosis of an ongoing pathological process is obtained. Not a genetic condition prodromal of the onset of solid tumors in a healthy subject.

Document US 2016/0102358 discloses a method of assessing an individual subject's risk of developing different types of cancer by identifying significant statistical association between multiple genetic markers and cancer risk for a variety of different cancers. In particular, the invention disclosed in US 2016/0102358 provides a method of producing a personalized cancer risk report for a subject by determining from a nucleic acid sample of the subject a genotype at a plurality of biallelic polymorphic loci. The risk is calculated based on the genotype determined for each plurality of biallelic polymorphic loci.

An article of GAGAN et Al, in "GENOME MEDICINE", Vol. 7, No. 1, 29 July 2015, pages 80-89, discloses methods for diagnosing and managing a patient having, for example, cytophenia. The methods include obtaining a sell-free DNA sample from peripheral blood plasma or serum of the patient, performing a mutation analysis of the of the cell free DNA sample on a panel of selected genes, and administering to the patient a treatment for management of a hematologic

malignancy.

Also the methods disclosed by GAGAN et Al. are directed to treatment of an already developed disease and does not teach nor suggest detecting a condition prodromal of the onset of solid tumors in a healthy subject.

The article of DE MATTOS-ARRUDA LETICIA et Al, in MOLECULAR ONCOLOGY, Vol. 10, No. 3, 17 December 2015, pages 464-474, discloses a diagnosis of cancer based on analysis of liquid biopsy while the article of MARK SAUSEN et Al., in NATURE COMMUNICATIONS, Vol. 6, No. 7686, 7 July 2015, pages 1-6, discloses a method to non-invasively scan tumor genomes and quantify tumor burden. Also in these documents the described methods are directed to treatment of an already developed disease and no teachings or suggestions are given for detecting a condition prodromal of the onset of solid tumors in a healthy subject.

Document US 2016/0130648 discloses methods for treating, managing, diagnosing and monitoring myelodysplastic syndrome and other hematologic malignancies. These methods comprise sequencing analysis conducted on cell-free DNA from peripheral blood plasma or serum. By the methods described in US 2016/0130648 diagnosis and post-diagnosis treatments are carried out with the purpose of finding and treating people that already have a cancer. No teachings or suggestions are given for detecting a condition prodromal of the onset of solid tumors in a healthy subject.

TECHNICAL PROBLEM

The evaluation of the risk status constitutes an essential component of the testing procedures and genetic analysis. There is a strong likelihood that in the very near future this type of approach will be implemented in a systematic manner in the prior assessment of the cancer pathologies.

On the other hand, it is more and more frequent that “healthy” persons ask to have genetic tests for predisposition to serious pathologies or detecting a pre-pathological status. Also for this reason the physicians should introduce techniques for the definition and management of risk, and for identifying surely or very probably pre-pathological conditions, in their routine monitoring programmes.

The genetic risk refers to the likelihood of an individual carrier of a mutation

associated specifically to a determined pathology - in particular as regards the present disclosure, a cancer pathology - actually developing this pathology. On the other hand, the identification of a pre-pathological condition or a condition prodromal of the onset of a pathology relates to the detection of biomolecular signals indicative of a genetic instability situation, which, due to a subsequent evolution, can cause over time, certainly or very likely, the onset of the pathology.

Given that, as already pointed out several times in the text, the carcinogenesis is influenced by both environmental factors and hereditary predisposition, the genetic background associated with a specific disorder considerably affects the definition of the risk correlated to this particular disorder.

According to Wang E. et al. an algorithm based on a series

(network) of characteristic elements can be adopted to generate a genetic model of the key components of the tumor and to connect mutating genotypes with clinical phenotypes. The use of this pattern (and others similar) illustrates the strategies for the prediction of possible tumor therapeutic targets, probability of recurrence and risk of the onset of the tumor on the basis of an individual profile of the patient's genomic sequence.

To sum up, prediction methods deriving from a model based on a network of characteristic elements can be used in the diagnosis and optimized management and prevention of the cancer pathologies programmes.

Genomic instability arises in normal cells through accumulation of genetic and epigenetic changes and has been shown to occur over a variable time span, ranging from years to decades (Gerlinger et al., 2012; Lengauer, Kinzler, & Vogelstein, 1998; Yates et al., 2015; Zhang et al., 2014).

The vast majority of normal cells that have acquired genomic instability features are thought to be cleared away by the immune system, while a minimal fraction might eventually progress and give rise to cancer (Nakad & Schumacher, 2016).

Upon development of cancer, specific genomic alterations can be identified and used to provide the rationale for specific treatment options. Monitoring genomic instability could hence be crucial to identify early mutational events that are associated with higher risk of developing cancer, but it is largely unfeasible using traditional tissue-based approaches. In contrast, liquid biopsy might offer the possibility of detecting early genomic aberrations and understanding cancer cells evolution, allowing the interrogation of cancer cells longitudinally and in a minimally invasive fashion. Liquid biopsy is a broad term that refers to the sampling of non-solid biological tissue, most commonly blood, but also saliva, urine and other body fluids. Interestingly, cancer patients typically present higher levels of total circulating cfDNA (cell-free DNA) compared to healthy individuals ((Alix-Panabieres & Pantel, 2016; Bettegowda et al., 2014).

The majority of DNA fragments in circulation, including cfDNA and ctDNA (circulating-tumor DNA), measures between 180 and 200 nucleotides in size. This observation suggests that cell death associated to physiological tissue remodeling events is likely to produce the majority of DNA fragments found in the bloodstream. Conversely, ctDNA is derived from tumor cells undergoing either apoptosis or necrosis and represents only a minor fraction (<0.1–10%) of the total circulating

cfDNA. A typical output for cf/ctDNA analysis is generated through the evaluation of genomic events in well-defined cancer-associated genes, often by using highly sensitive technologies as digital PCR or next-generation sequencing (NGS) {Goodwin:2016fy}. ctDNA analysis has been applied to monitor response to therapy, to detect minimal residual disease and to assess the development of resistance to therapy (Diaz et al., 2012; Siravegna et al., 2015; Tokudome & Hayes, 2013).

Currently, the most common clinical use of liquid biopsy is monitoring tumor burden in response to therapy and the identification of resistance-associated genetic alterations to inform treatment decision ((Azad et al., 2015; Fribbens et al., 2016; Misale et al., 2012; Weigelt et al., 2017).

The introduction of molecular barcodes has considerably enhanced the sensitivity of sequencing methods (down to 0.01% of variant allelic frequency), at the price of additional costs given the extremely high level of sequencing depth required (i.e., ~25,000 coverage) ((Kinde, Wu, Papadopoulos, Kinzler, & Vogelstein, 2011; Newman et al., 2016; Schmitt et al., 2012).

Taking advantage of this and further technological advances, a number of studies have described clinically-relevant genetic alterations in patients with early-stage cancers, even at a sensitivity of <1 mutant template molecules per milliliter of plasma (Abbosh et al., 2017; Aravanis, Lee, & Klausner, 2017; Bettegowda et al., 2014; Cohen et al., 2017; 2018)

Nevertheless, among the potential clinical applications of ctDNA analysis, early detection remains the most ambitious, given the substantial number of patients and healthy controls necessary to establish the required sensitivity and specificity for such testing approach (De Mattos-Arruda et al., 2014).

Furthermore, age-related clonal hematopoiesis in asymptomatic patients might be as well a source of false positive results (Cohen et al., 2018), a phenomenon not entirely understood in the general population but well-established in patients that survived (i.e. considered clinically cured) hematological cancers (Cree et al., 2017; Genovese et al., 2014).

Importantly, monitoring overall genomic instability in healthy individuals could lead to the identification of subpopulations that might enter into early access screening prevention programs. Nevertheless, the recovery and characterization of cfDNA in healthy individuals might prove challenging, given that cfDNA is less abundant in these subjects, and that there are only few studies describing cfDNA

analysis in healthy controls (Cohen et al., 2018; Cree et al., 2017). It is intuitive, therefore, that the above-described model could only be successful after an adequate technical validation, proving that extraction and analysis of cfDNA isolated from healthy individuals is technically achievable. To this end in our proof of principle study, we investigated the possibility to interrogate cfDNA in a collection of clinically healthy individuals (i.e. not affected by any manifest medical condition) at the time of blood collection. First, we evaluated the reliability of our testing strategy by analyzing cfDNA samples obtained from patients with an histologically confirmed diagnosis of breast or lung cancer. Specifically, we assessed the mutational status concordance in matched tissue and plasma specimens. We then analyzed a group of healthy donors comprising both individuals that did not develop any tumor within 1 to 10 years (average = 8.39 years, Table 1) of follow-up as well as individuals that developed either a benign neoplasm or cancer during the follow-up time. Altogether, our study demonstrates the technical feasibility of extracting and analyzing cfDNA in healthy individuals to study genomic alterations, by means of molecular barcoded ultra-deep sequencing.

OBJECTS OF THE INVENTION

The main object of the invention is to provide a monitoring method capable of obtaining an evaluation, for a clinically healthy individual, of his/her entry into a genetic condition prodromal of the onset of one form of solid tumor, that is a condition that, sooner or later, almost certainly will make the individual develop the form of tumor being monitored.

SUMMARY OF THE INVENTION

This and other objects are obtained by the invention, which relates to a method (based on an algorithm and an associated database) for searching and identifying, in a healthy individual, a genetic condition that is prodromal of development of solid tumors, with the exception of the brain tumors. This method defines the above mentioned condition on the basis of a series of periodical evaluation cycles about the mutation frequency that involve a panel of genes chosen from those associated to the onset of the cited solid tumors.

The method includes taking of a biological sample from an individual (constituted by body fluids, such as blood, urine or spinal

CLAIMS

1. A method for searching and identifying a genetic condition prodromal of the onset of solid tumors in a healthy subject, characterized by including an evaluation cycle for the evaluation of genetic stability or instability condition and at least one repetition cycle of such evaluation, said repetition cycles being periodically performed on the subject, with each cycle comprising the steps of:
   - taking a sample of biological material of the subject, isolating DNA from the biological material, amplifying and sequencing the isolated DNA;
   - verifying the presence of mutations selected in a predetermined set of genes of the sample under consideration, said set of genes and said mutations being associated with the onset of solid tumors;
   - the predetermined set of genes and selected mutations are defined in view of the subject's anamnesis;
   - the predetermined set of genes including either a subset of the panel of genes or hotspots connected to one or more solid tumors, or the entire panel of genes connected to solid tumors;
   - verifying the frequency of mutations detected for each gene and for each evaluation cycle, the mutations being chosen from the aforementioned selected mutations;
   - recording the mutations detected for each gene or group of genes and their frequency;
   - defining or updating a genetic instability index of the subject, either overall (I_T,) or for a single gene (I_G), for each repetition cycle, based on the frequency of mutations detected and on the basis of the increase in the frequency of mutations said genetic instability index (I_T,I_G) is also defined on the basis of the increase in the frequency of mutations with respect to one or more previous evaluation cycles;
   - evaluating, in each repetition cycle, the subject's entry into a genetic condition prodromal of the onset of one or more solid tumors or groups of solid tumors on the basis of a threshold value (I_{TS},I_{GS}) of said genetic instability index (I_T,I_G), defined for

each single gene or group of genes, being exceeded.

2. The method of claim 1, wherein said overall genetic instability index (I_T) is defined as the summation of the relationships between a value (ΔF_k) responsive to the increase in the observed mutations for each gene and the number of genes evaluated in consideration of the whole group of monitored genes.
3. The method of claim 1, wherein said index of genetic instability for single gene (I_G) is defined as the summation of the relationships between a value (ΔF_j) responsive to the increase in the observed mutations for each hotspot and the number of hotspots evaluated, in consideration of the whole group of monitored hotspots.
4. The method of claim 1, wherein the biological sample consists of a liquid biopsy, and the phase of verification of the presence of mutations in the predetermined set of genes is performed on a DNA sample isolated from said liquid biopsy and subsequently amplified and sequenced.
5. The method of claim 4, wherein the liquid biopsy is peripheral blood.
6. The method of claim 4, wherein the liquid biopsy is urine or spinal fluid.
7. A method according to one of the claims 4 to 6, wherein a fraction of cfDNA is sought in the DNA sample being analyzed and wherein the presence of mutations is verified in said cfDNA fraction.
8. A method according to one of the claims 4 to 6, wherein the ctDNA isolated from the liquid biopsy is also sought in the DNA sample being analyzed.
9. A method of claim 8, wherein, following the identification of circulating ctDNA, addressing information is sent to an early detection system ("*Early Detection*").
10. The method of claim 8, wherein, following the identification of ctDNA in said DNA sample being analyzed, the presence of CTC ("*Circulating Tumor Cells*") is sought in said liquid biopsy.
11. The method of claim 1, wherein the predetermined set of genes and selected

mutations are defined in view of their connection to particular types of tumor.

12. A method of claim 1, wherein the repetition period is recalculated after each repetition cycle according to a value of said instability index (I_T,I_G) calculated in the current cycle and a value of the same as calculated in one or more previous cycles.
13. A method of claim 1, wherein a greater analysis sensitivity related to the monitored gene or panel of genes is set in each repetition cycle, following an increase in the calculated value for said instability index (I_T,I_G) with respect to the value of the same index (I_T,I_G) calculated in one or more previous cycles.
14. The method of claim 1, wherein, in each of said evaluation and repetition cycles, the germ DNA is also isolated and sequenced, and only the mutations present in the cfDNA and not present in said germ DNA are considered for the subsequent calculation of the instability index.
15. The method according to claim 14, wherein said germ DNA is sequenced with the same reading degree of the sequencing of the cited cfDNA.
16. The method of claim 14, wherein said germ DNA derives from the same liquid biopsy from which the cited cfDNA has been taken.
17. A system for searching and identifying a genetic condition prodromal of the onset of solid tumors in a healthy subject, comprising a set of instructions of computer program aimed at carrying out an evaluation cycle of a genetic stability or instability condition and at least one repetition cycle of said evaluation defined according to claim 1.