

For example, Chen et al.⁴⁵ found ECT2 expression is closely associated with the activation of the Rho/ERK signaling axis in promoting early HCC recurrence. Sun et al.⁴⁶ has also demonstrated that another gene, PLK1, was expressed significantly higher in HCC sample and can be an independent prognostic factor for HCC applicable for diagnosis and treatment. Similar conclusions were drawn by Takai et al.⁴⁷ in their publication supporting findings of Sun et al.⁴⁶. Researchers have further devised several diagnostic or prognostic means capitalizing on the association established between liver cancer and PLK1 or ECT2 expression. For instance, United States patent publication no. 2011/03119280 discloses a DNA biochip for HCC diagnosis by scanning multiple gene markers including PLK1. Most of the aforesaid approaches centered on cancer prognosis correlating to individual expression of the candidate genes or proteins instead of in-depth information generated according to correlation between or among the candidate genes or proteins, though derivation of diagnostic outcome based upon interrelationship of multiple expressed candidate genes has been proposed in some publications relating to other cancer types. Somma et al. in their United States patent publication no. 2012/0197540 provide a method to predict mortality risk of a breast cancer patient by measuring expression level of multiple genes including both PLK1 and ECT2 in a tissue sample of the cancer patient; the method subsequently employs each of the measured genes expression level to compute a prognostic score, through a predetermined algorithm, being indicative of the mortality risk of the subject. However, no similar method or platform exploiting on interrelationship of co-expressed genes or protein for prognostic or diagnostic of liver cancer have been disclosed. The prognostic outcome derived from such method or platform should be applicable to facilitate better therapeutic results or allotment of medical resources.

Summary

The present disclosure is directed to a method for measuring or quantifying expression of PLK1 and ECT2 in a tissue specimen or sample acquired from a subject. Particularly, the expression described herein can refer to at least one of transcriptome or protein expression in the specimen. Preferably, the tissue sample is of HCC- or CCA-affected.

Thus, it might be possible to offer a method for generating a prognostic or diagnostic result regarding a subject affected by HCC or CCA using the measured or quantified PLK1 and ECT2 expression in a tissue specimen. The prognostic or diagnostic result may generally relate to predictive survival outcome or mortality risk of the subject. The prognostic or diagnostic result

can be referred by a physician for carrying out precision medicine. Further, the present disclosure may provide an apparatus being configured to, preferably in a fully or semi-automated fashion, quantify expression of PLK1 and ECT2 then generate the prognostic or diagnostic results with respect to medical condition of a subject affected by liver cancer.

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Aspects of the invention are defined in the independent claims. Some optional features of the invention are defined in the dependent claims.

At least one of the preceding exemplary arrangements can be met, in whole or in part, by the present techniques, in which one of the examples includes a method for generating a predictive survival outcome of a subject affected by liver cancer, comprising: providing a tissue specimen of the subject, the tissue specimen including or being one of hepatocellular carcinoma or cholangiocarcinoma; performing at least one nucleic acid-based and/or proteomic-based assay to quantify transcriptome and/or protein expression of polo-like kinase 1 (PLK1) and epithelial cell transforming 2 (ECT2) in the provided specimen; deriving a ratio of PLK1 expression over ECT2 expression within a defined area of the provided specimen; and generating the predictive survival outcome of the subject according to the derived ratio, the subject being regarded with a first predictive survival outcome when the derived ratio is less than a predetermined value or a second predictive survival outcome when the derived ratio is equal to or greater than the predetermined value. More particularly, the first predictive survival outcome corresponds to more than 40%-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40%-60% or lower of overall survival percentage within the predetermined period in the survival analysis. Typically or preferably, the predetermined period is, but not limited to, 12 to 36 months.

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In a number of examples, the nucleic-acid based assay is any one or combination of microarray-based technologies and polymerase chain reaction based technology including digital PCR.

In several examples, the proteomic-based assay includes or is a set of tissue microarrays and immunohistochemistry staining of human tissues.

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In another aspect of the present disclosure, an apparatus for generating a predictive survival outcome of a subject having liver cancer is disclosed. The apparatus comprises a first module

capable of identifying, detecting or determining transcriptome and/or protein expression of polo-like kinase 1 (PLK1) and epithelial cell transforming 2 (ECT2) in a tissue specimen of the subject; and a second module being configured for deriving a ratio of PLK1 expression over ECT2 expression within a defined area on the provided specimen and generating the predictive survival outcome of the subject according to the derived ratio, the second module configured to regard the subject as having a first predictive survival outcome when the derived ratio is less than a predetermined value, or having a second predictive survival outcome when the derived ratio is equal to or greater than the predetermined value. More specifically, the first predictive survival outcome corresponds to more than 40-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40-60% or lower of overall survival percentage within the predetermined period in the survival analysis. Typically or preferably, the nucleic-acid based assay abovementioned can refer to any one or combination of microarray-based technologies such as microarray and polymerase chain reaction based technology including digital PCR. Likewise, the proteomic-based assay can be tissue microarrays and immunohistochemistry staining of human tissues.

For a plurality of examples, the disclosed apparatus further comprises a test module capable of performing at least one nucleic acid-based or proteomic-based assay to couple expressed transcriptome or protein of PLK1 and ECT2 with a signaling moiety fashioned to continuously or periodically emit a signal detachable by the first module.

In some examples, the subject is preferably of Asian descent to attain results of greater accuracy. Also, the tissue specimen of the subject includes or is carcinoma being one of hepatocellular carcinoma or cholangiocarcinoma.

Brief Description of Drawings

Figure 1 shows results of consensus clustering with a hierarchical clustering algorithm with left panel: Empirical cumulative distribution (CDF) plots corresponding to the consensus matrices in the range of cluster number (K) = 2-8, middle panel: Corresponding change in area under CDF, right panel: Consensus matrices corresponding to a number K of clusters ranging between 2 and 5. Results for CCA or HCC samples are shown on the top or bottom row, respectively;

Figure 2 (a) is a heatmap of CCA subtypes based on consensus clustering and hierarchical clustering of the most variable genes that the x-axis represents CCA subtype consensus clusters, with CCA samples being represented in columns, grouped by the dendrogram into 4 main clusters and genes being represented in rows, the

subject being regarded with a first predictive survival outcome when the derived ratio is less than a predetermined value or a second predictive survival outcome when the derived ratio is equal to or greater than the predetermined value. Preferably, the first predictive survival outcome corresponds to more than 40%-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40%-60% or lower of overall survival percentage within the predetermined period in the survival analysis. It was found by the inventors of the present disclosure that relative expression of PLK1 and ECT2, tumor markers, in diseased tissue carrying or affected with hepatocellular carcinoma or cholangiocarcinoma correlates to survival outcome of the tested subject or acuteness of the liver cancer adversely affecting the subject. The acquired predictive survival outcome can be an effective tool to facilitate planning of strategic treatment and corresponding arrangement of medical resources according to medical needs of the tested subject.

According to several exemplary arrangements, the tissue specimen affected with hepatocellular carcinoma or cholangiocarcinoma in the present disclosure can be acquired through any operation(s) or medical procedure(s) known in the field. Preferably, the tissue specimen is extracted by way of liver biopsy, which can be, but is not limited to, a percutaneous-, transjangular- or laparoscopic-based procedure. For a number of exemplary arrangements, the type of liver biopsy carried out is dependent upon the downstream assay for quantifying the expression of transcriptome and/or protein relating to PLK1 and/or ECT2. For instance, the tissues specimen acquired through laparoscopy is more suitable for proteomic-based assay such as tissue microarray requiring sufficient area in the specimen to be prepared for immunochemistry staining and signal detection. On the other hand, it is preferable to subject tissue specimen obtained from percutaneous biopsy for nucleic acid-based assay that the mRNAs or transcriptomes corresponding to expression of PLK1 and/or ECT2 extracted from the specimen, proportionally amplified, and finally quantified.

For a number of examples, the nucleic-acid based assay is any one or a combination of microarray-based technologies and polymerase chain reaction based technology including digital PCR. The nucleic-acid based assay generally involves extraction of the overall mRNAs or transcriptomes from the tissue specimen of predetermined dimension followed by selectively quantifying the mRNAs or transcriptomes relating to PLK1 and/or ECT2 expression, with or without selective amplification of the mRNAs or transcriptomes of PLK1 and/or ECT2. Preferably, the

transcriptomes of PLK1 and/or ECT2 hybridize on substantially complementary probes anchored on a platform. The hybridization process is configured to emit a signal, preferably a light signal, detectable by one or more sensors located approximate to or within the platform. The probes are tagged with a signaling moiety which is fashioned to generate the light signal upon hybridization and forming of the double-stranded nucleic acids. The signals emitted are preferably proportional to the number of hybridization reactions that have occurred in the platform that enable subsequent quantification of the PLK1 and/or ECT2 expression by referring to a pre-established standard.

Similarly, for a plurality of examples, the proteomic-based assay is tissue microarrays and immunohistochemistry staining of human tissues by which the expressed protein or peptides of PLK1 and/or ECT2 are selectively coupled to one or more signaling moieties succeeding by detecting the signaling released from the coupled signaling moieties to result in quantification of the PLK1 and/or ECT2 expression. For example, in tissue microarrays (TMAs), the acquired tissue sample is used to prepare 0.5-2.0 mm cores of formalin fixed and paraffin embedded tissue sample.

The prepared tissue sample proceeds to immunohistochemistry treatment at 1 to 10 μ m TMA sections in the form of slides. Further, the slides containing trimmed segment of the tissue specimen are deparaffinized in xylene, and rehydrated in graded alcohol. Antigen retrieval is then performed under pressure for 5 to 60 minutes with an acidic buffer. Anti-PLK1 (mouse monoclonal) is applied to the treated tissue sample at a 1:1000 dilution in a 2% non-fat milk solution at room temperature for 1 to 4 hours. Anti-ECT2 (mouse monoclonal) is applied at a dilution of 1:500 in a 2% non-fat milk solution. By bringing the antibody into contact with the targeted tumor marker or protein, the disclosed method results in yielding of antigen-antibody complexes detachable through a sensor or system such as those commercially available, for instance, Envision+ system (Dako) with concurrent use of 3,3'-diaminobenzidine (DAB).

Preferably, the slides are counterstained with hematoxylin, dehydrated, cleared and coverslipped. Detection of the tagged or stained PLK1 and/or ECT2 can be performed at 200X or higher magnification for quantification of the PLK1 and/or ECT2 expression within a defined area. It is worth noting that the quantification can be performed manually by trained personnel or with the assistance of a signal sensor or image analyzer coupled to a computing device. Preferably, the treated tissue specimen can be scored for various image parameters or properties such as percentage of tumor cells and intensity of the indicating signal or staining. The scores of different image properties generated can be further manipulated such as by way of multiplying each other or being input into one or more algorithms to yield more insightful findings with respect to the

subject affected by hepatocellular carcinoma or cholangiocarcinoma. In a number of examples, the expression level of PLK1 or ECT2 in the disclosed method corresponds to or is defined by multiplying scores acquired from the treated specimen or image captured for the treated specimen. The expression level can be quantified by way of multiplying a score of percentage of tumor cells
5 expressing PLK1 or ECT2 with a score of intensity of the signal or staining of the expressed PLK1 or ECT2, providing a computed score substantially representing the expression level of each tumor marker. For such examples, the expression level of the tumor marker under consideration corresponds to the computed score. The scale of the scores for each image property can range from 1-15, more preferably 1-10, and most preferably 1-4. Multiple areas of the treated specimen are
10 scored independently; in some examples that the disclosed method computes a mean using these independent scores to attain better representation of the PLK1 and/or ECT2 expression, potentially yielding results of greater accuracy.

With the expressed transcriptomes and/or protein detected and quantified through the nucleic acid-
15 based or protein-based assay, the disclosed method sets out to derive the ratio of the PLK1 expression over ECT2 expression within a defined area of the provided specimen. For example, the derived ratio is 1.5 when the expression level of the PLK1 is numerally represented as 12 and the expression level of ECT2 calculated is 8. For several examples, the ratio can be calculated or computed by inverting the numerator and denominator of the aforesaid ratio employing ECT2 and
20 PLK1 expression levels respectively as numerator and denominator instead. Preferably, the area has a dimension of, but not limited to, 0.1 to 1.0mm in length, 0.1 to 1.0mm and 1 to 4um in thickness. To simplify the process in deriving the predictive survival outcome of the subject, the disclosed method regards the subject to have the first predictive survival outcome when the derived ratio is less than a predetermined value or the second predictive survival outcome when
25 the derived ratio is equal to or greater than the predetermined value. In various examples, the predetermined value can be any number within, but not limited to, the range of 0 to 16 in connection to a 0-4 scale of scores used. The predetermined number can range from 0.001 to 1 as well depending upon the scale of scores applied in the relevant examples. Also, the derived ratio can be analyzed or mathematically further associated with expression level of other genes or
30 proteins relating to hepatocellular carcinoma or cholangiocarcinoma to produce a further or more conclusive prediction or observation.

As aforementioned, in accordance with some examples, the first predictive survival outcome corresponds to more than 40%-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40%-60% or lower of overall survival percentage within the predetermined period in the survival analysis. In addition, the survival percentage generated can be considered as an indicator offering empirical information with respect to potential responsiveness of the tested subject towards general treatment against hepatocellular carcinoma or cholangiocarcinoma. The tested subject may have to undergo alternative treatment option rather than conventional remedy when the subject has been associated with or assigned to second predictive survival outcome or relatively poor survival percentage. The inventors of the present disclosure also noticed that the disclosed method yields more reliable or robust results for those subjects with Asian genetic makeup or Asian descent.

In some examples, the predictive survival outcome portrays or only portrays survival percentage of the subject over a predetermined period which preferably spans for 6 to 48 months, or more preferably 12 to 36 months.

Pursuant to another aspect of the present disclosure, an apparatus for generating a predictive survival outcome of a subject affected of liver cancer is disclosed. Particularly, the disclosed apparatus comprises a first module capable of identifying or detecting transcriptome and/or protein expression of PLK1 and ECT2 in a tissue specimen of the subject; and a second module being configured to derive a ratio of PLK1 expression over ECT2 expression within a defined area on the provided specimen and generating the predictive survival outcome of the subject according to the derived ratio, the second module being configured to regard the subject to have a first predictive survival outcome when the derived ratio is less than a predetermined value or regard the subject to have a second predictive survival outcome when the derived ratio is equal to or greater than the predetermined value. Preferably, the first predictive survival outcome corresponds to more than 40%-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40%-60% or lower of overall survival percentage within the predetermined period in the survival analysis.

The first module described in the present disclosure can be any known platform operable under the principles of genetic, proteomic, immunochemistry and/or histochemistry to selectively render PLK1 and/or ECT2 expression detectable by human or machine reading. Therefore, in some

examples, the disclosed apparatus may further comprises a test module capable of performing at least one nucleic acid-based or proteomic-based assay to couple expressed transcriptome or protein of PLK1 and ECT2 with a signaling moiety fashioned to continuously or periodically emit a signal detachable by the first module. Particularly, the first module is configured to attach or couple the transcriptomes and/or proteins expressed for PLK1 and/or ECT2 with a signaling moiety capable of releasing detectable signal to highlight presence of the expressed transcriptomes or protein to which the signaling moiety is coupled to. The first module of the present disclosure may be composed of multiple working stations arranged to run sequential processes to prepare and treat the tissue specimen to finally lead to identification of the PLK1 and/or ECT2 expression. These multiple working stations can be interconnected or operate separately one another according to the preference of the disclosed examples. Preferably, in more examples, the first module comprises an illumination device for the first module to highlight the expression of PLK1 and/or ECT2, and an image capture device designed to create a digital image of the illuminated specimen; the first module uploads the taken image for the second module to analyze and derive the predictive survival outcome. Most preferably, the first module stores the created digital image in a database in communication such that the stored image can be recalled and subjected to further analysis. For instance, the first module can be gene expression microarray or tissue microarray system for detecting and quantifying expression of the PLK1 and/or ECT2 in tissue sample affected of hepatocellular carcinoma or cholangiocarcinoma.

According to a number of examples, the second module is adapted to derive the ratio of PLK1 expression over ECT2 expression or vice versa within the defined area of the provided specimen and generating the predictive survival outcome of the subject correlating to the derived ratio. The second module can be a computing means installed with a set of commands in the form of computing program to run the analysis on the captured image, properties of the captured image may be correspondingly manipulated in line with the analysis performed. Preferably, the second module analyzes the treated tissue specimen, or more preferably image of the treated tissue specimen, and provides score for various image parameters or properties such as percentage of tumor cells and intensity of the indicating signal or staining shown. The second module may further manipulate scores of different image properties generated such as multiplying the scores to each other or input the scores into one or more algorithm to yield more insightful finding with respect to the subject affected by hepatocellular carcinoma or cholangiocarcinoma. Still, in more examples, the second module defines expression level of PLK1 or ECT2 in a numeral computed

score resulted from different scores individually associated with a plurality of properties in the image captured for the treated tissue specimen. For example, the second module quantifies the expression level by way of multiplying score of percentage of tumor cells expressing PLK1 or ECT2 as detected with score of intensity of the signal or staining of the expressed PLK1 or ECT2, yielding the computed score substantially representing the expression level of each targeted tumor marker. For such examples, the expression level of the interested tumor marker is regarded by the disclosed apparatus corresponding to the computed score. The scale of the scores for each image property can range from 1-15, more preferably 1-10, and most preferably 1-4. The second module generates individual score for multiple areas of the treated specimen in some examples that the second module computes a mean of these independent scores to attain better representation of the PLK1 and/or ECT2 expression in the captured image.

Furthermore, the second module computes or derives the ratio of the PLK1 expression over ECT2 expression, or vice versa, within a defined area of the provided specimen. Preferably, the area has a dimension of 0.1 to 1.0mm in length, 0.1 to 1.0mm and 1 to 4um in thickness. To simplify the process in deriving the predictive survival outcome of the subject, the disclosed second module is regards the subject to have the first predictive survival outcome when the derived ratio configured to regard the subject to have a first predictive survival outcome when the derived ratio is less than a predetermined value or regard the subject to have a second predictive survival outcome when the derived ratio is equal to or greater than the predetermined value. The predetermined value can be any number, but not limited to, within the range of 0 to 16 depending on the scale of score implemented. As abovementioned, the first predictive survival outcome corresponds to more than 40%-60% of overall survival percentage within a predetermined period in a survival analysis, and the second predictive survival outcome corresponds to 40%-60% or lower of overall survival percentage within the predetermined period in the survival analysis. Preferably, the predetermined period preferably spans for 6 to 48 months, or more preferably 12 to 36 months.

For some examples, the nucleic-acid based assay is any one or combination of microarray-based technologies for DNA and/or RNA polynucleotides detection, and polymerase chain reaction based technology including digital PCR.

In other examples, the proteomic-based assay is tissue microarrays tissue microarrays and/or immunohistochemistry staining of human tissues.

The following example is intended to further illustrate the invention, without any intent for the invention to be limited to the specific embodiments described therein.

Example 1

5 *Cohorts and Clinical Specimens*

A set of 398 surgical paired tumor and nontumor specimens derived from 199 sequential patients of the TIGER-LC cohort (130 CCA patients and 69 HCC patients) were used in this study. Tumor