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(54) **Title:** NEW BIOMARKERS FOR ACUTE MYELOID LEUKEMIA

(57) **Abstract:** The present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value. The invention also relates to an i) inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML).



NEW BIOMARKERS FOR ACUTE MYELOID LEUKEMIA

FIELD OF THE INVENTION:

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The present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

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The invention also relates to an i) inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML).

BACKGROUND OF THE INVENTION:

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Acute myeloid leukemia (AML) is a heterogeneous disease characterized by different recurrent cytogenetic and molecular aberrations occurring in hematopoietic progenitor cells altering their growth, differentiation and proliferation which provide the most important prognostic information at diagnosis. The treatment of AML has remained a huge challenge for onco-hematologists. Although roughly 70% of adults with AML under age 60 achieve a complete remission with traditional cytarabine- and anthracycline- based induction regimens, the overall long-term survival rate with therapy continues to be insufficient at approximately 30% to 40%. The prognosis is even worst for older patients, for which long-term survival is less than 10%. Whereas recurrent chromosomal structural alterations are well-established prognostic markers, the outcome of patients with a normal karyotype has been recently refined with the successive discoveries of recurrent mutations in the FLT3, NPM1, CEBPA genes. Indeed, these genetic alterations have been associated with outcome after intensive chemotherapy and now serve as a basis for molecularly guided risk assessment and treatment stratification [Dohner H. et al, 2010]. In addition, modulated expression of genes such as

MN1, BAALC, ERG, and WT1 were also demonstrated to be predictive for outcomes of patients with cytogenetically-normal AML (CN-AML) [Hermkens MC et al., 2013; Heuser M et al., 2006; Santamaria C et al., 2010]. However, the role of most of these genes in leukemogenesis remains unclear and they offer poor opportunity for drug targeting.

5 The inventors reasoned that DNA replication represents an under-explored source of prognostic markers that could be used in combination with cytogenetics to predict AML prognosis and eventually provide valuable druggable targets. Indeed faithful execution of the spatio-temporal DNA replication program is a cardinal step to limit cancer risk through the preservation of genome integrity. Multiple evidences have accumulated in solid cancers
10 indicating that alterations of the DNA replication program trigger replicative stress favoring the accumulation of genetic alterations [Allera-Moreau C et al., 2012; Bartkova J et al., 2005; Gorgoulis VG et al., 2005; Lemee F et al., 2010; Pillaire MJ et al., 2010]. Surprisingly, DNA replication defect as a source of markers in hematological malignancies has been totally under-explored. Recently, genome-wide replication-timing analyses detected widespread
15 deregulation of replication timing in leukemic cells, all sharing specific replication-timing aberrations, indicating common early replication defect events in leukemogenesis that appear to be conserved [Ryba T et al., 2012]. The inventors hypothesized here that mis-expression of replication genes affecting DNA replication program could occur during AML leukemogenesis, contributing to the cytogenetic aberrations that characterize AML. They
20 speculated that specific replication control gene signatures could be highly relevant during relapses, representing potential predictors for outcomes of patients with AML. They also reasoned that a modified replication control could impact on the response to the backbone of AML treatment since several decades, the nucleoside analog cytarabine, a well-known inhibitor of DNA chain elongation in the course of the replication fork progression.

SUMMARY OF THE INVENTION:

By using a series of 198 AML patients treated by intensive chemotherapy, the inventors find that CHK1 and POLQ genes implicated in the DNA replication control of cells,
30 can be used as biomarkers for the survival time of a patient suffering from acute myeloid leukemia and treated with traditional cytarabine and anthracycline based induction regimens.

Thus, the present invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene

expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

The invention also relates to an i) inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML).

DETAILED DESCRIPTION OF THE INVENTION:

Predictive methods

A first aspect of the invention relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

In one embodiment, the expression level of the gene CHK1 is determined.

In one embodiment, the expression level of the gene POLQ is determined.

In one embodiment, the expression levels of the 2 genes CHK1 and POLQ are determining in the same time.

Thus, the invention also relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the gene expression levels of the genes CHK1 and POLQ ii) comparing the gene expression levels determined at step i) with their predetermined reference values and iii) providing a good prognosis when the expression levels determined at step i) are all lower than their predetermined reference values, providing a bad prognosis when the expression levels determined at step i) are all higher than their predetermined reference values and providing an intermediate prognosis when the

expression level of one gene is lower than its predetermined value and when the expression level of the other gene is higher than its predetermined value.

With respect to intermediate prognosis, every time that the expression level of a gene is lower than its predetermined reference value, the more favourable will be the prognosis of the patient.

In another embodiment, the invention, the invention relates to a method for predicting the overall survival (OS) of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

In another embodiment, the invention, the invention relates to a method for predicting the event-free survival (EFS) of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

In another embodiment, the invention, the invention relates to a method for predicting the relapse free survival (RFS) of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the expression level determined at step i) is higher than its predetermined reference value.

As used herein and as explained in the examples, “Complete Response (CR)” required a normocellular bone marrow with less than 5% blasts and no Auer rods, neutrophil count $\geq 1 \times 10^9/L$ and platelet count $\geq 100 \times 10^9/L$, without evidence of extramedullary disease after one or two courses of treatment. Induction failures included deaths in aplasia and resistant disease.

5 Overall survival (OS) was calculated from the date of the first day of chemotherapy until the date of death from any cause. Event-free survival (EFS) was calculated from the first day of chemotherapy until the date of treatment failure, relapse, or patient death from any cause. Relapse-free survival (RFS) for patients who achieved CR was calculated from the date of CR until the date of relapse or death from any cause with censoring of other patients at the date of
10 last follow-up.

As used herein, the term “Overall survival (OS)” denotes the percentage of people in a study or treatment group who are still alive for a certain period of time after they were diagnosed with or started treatment for a disease, such as AML (according to the invention). The overall survival rate is often stated as a five-year survival rate, which is the percentage of
15 people in a study or treatment group who are alive five years after their diagnosis or the start of treatment.

As used herein, the term “Event-Free Survival (EFS)” denotes the length of time after primary treatment for a cancer ends that the patient remains free of certain complications or events that the treatment was intended to prevent or delay. These events may include the
20 return of the cancer or the onset of certain symptoms, such as bone pain from cancer that has spread to the bone.

As used herein, the term “Relapse Free Survival (RFS)” denotes the length of time after primary treatment for a cancer ends that the patient survives without any signs or symptoms of that cancer.

25 As used herein, the term “Good Prognosis” denotes a patient with more than 50% chance of survival for the next 5 years after the treatment.

In another aspect, the invention relates to a method for predicting the responsiveness of a patient affected with an acute myeloid leukemia (AML) to a cytarabine and/or anthracycline treatment comprising i) determining in a sample obtained from the patient the
30 gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value wherein when the gene expression level determined at step i) is lower than its predetermined reference values then the responsiveness of the patient to the treatment is good,

and when the gene expression level determined at step i) is higher than its predetermined reference value then the responsiveness of the patient to the treatment is bad.

In one embodiment, the expression level of the gene CHK1 is determined.

In one embodiment, the expression level of the gene POLQ is determined.

5 In one embodiment, the expression levels of the 2 genes CHK1 and POLQ are determining in the same time.

Thus, the invention also relates to a method for predicting the responsiveness of a patient affected with an acute myeloid leukemia (AML) to a cytarabine and/or anthracycline treatment comprising i) determining in a sample obtained from the patient the gene expression
10 levels of the genes CHK1 and POLQ ii) comparing the gene expression levels determined at step i) with their predetermined reference value wherein when the gene expression levels determined at step i) are all lower than their predetermined reference values then the responsiveness of the patient to the treatment is good, when the gene expression levels determined at step i) are all higher than their predetermined reference value then the
15 responsiveness of the patient to the treatment is bad and when the gene expression level determined at step i) for one gene is lower and for the other gene is higher than their predetermined reference values then the responsiveness of the patient to the treatment is intermediate.

With respect to an intermediate responsiveness, every time that the expression level of
20 a gene is lower than its predetermined reference value, the more favourable will be the response of the patient.

In one embodiment and according to the methods of the invention, the determination of the gene expression level of CHK1 and/or POLQ may be effected before or after the
25 beginning of the treatment (by cytarabine and/or anthracycline) of the patient.

In another embodiment, the patient affected with an AML is treated with cytarabine and anthracycline.

In another embodiment, the patient affected with an AML is not treated with cytarabine and anthracycline.

30 In another embodiment, the patient suffering from acute myeloid leukemia (AML) may be treated with another chemotherapy than cytarabine and anthracycline. For example, the patient may be treated with a chemotherapeutic agent targeting DNA.

Examples of chemotherapeutic agents targeting DNA replication include but are not limited to alkylating agents platinum analogs such as cisplatin and carboplatin; Hydroxyurea, 5-FluoroUracile, chain terminators such as AZT or dideoxydNTPs.

5 As used herein and according to all aspects of the invention, the term “CHK1” for “replication checkpoint 1” also known as CHEK1 refers to the human gene encoding a DNA replication checkpoint kinase that signals the DNA replication fork stalling and phosphorylates cdc25, an important phosphatase in cell cycle control, particularly for entry into mitosis (Entrez Gene ID number: 1111; mRNA sequence reference: NM_001114121; 10 protein sequence reference: NP_001107593). In addition, the invention encompasses all the isoforms of the said CHK1 gene. Sequences of all isoforms of CHK1 may also be found with the reference Hs00967506_m1 (Applied Biosystem). Isoform, as used herein, refers to all the different forms of the CHK1 gene and may be produced by mutations, or may arise from the same gene by alternative splicing. A large number of isoforms are caused by single nucleotide 15 polymorphisms or SNPs, small genetic differences between alleles of the same gene. These occur at specific individual nucleotide positions within a gene. Also included within this definition is the situation where different versions of messenger RNA are created from the same gene by employing different promoters, which causes transcription to skip certain exons. Thus, it is understood that the methods of the invention are not restricted to CHK1 per se, but also encompass one or several CHK1 isoforms. According to methods of the 20 invention, the expression level of the CHK1 gene and/or one or several of its isoforms are measured.

As used herein and according to all aspects of the invention, the term “POLQ” refers to the human gene encoding the DNA polymerase theta (Entrez Gene ID number: 10721; 25 mRNA sequence reference: NM_199420.3; protein sequence reference: NP_955452.3). In addition, the invention encompasses all the isoforms of the said POLQ gene. Sequences of all isoforms of POLQ may also be found with the reference Hs00198196_m1 (Applied Biosystem). Isoform, as used herein, refers to all the different forms of the POLQ gene and may be produced by mutations, or may arise from the same gene by alternative splicing. A 30 large number of isoforms are caused by single nucleotide polymorphisms or SNPs, small genetic differences between alleles of the same gene. These occur at specific individual nucleotide positions within a gene. Also included within this definition is the situation where different versions of messenger RNA are created from the same gene by employing different promoters, which causes transcription to skip certain exons. Thus, it is understood that the

methods of the invention are not restricted to POLQ per se, but also encompass one or several POLQ isoforms. According to methods of the invention, the expression level of the POLQ gene and/or one or several of its isoforms are measured.

5 As used herein and according to all aspects of the invention, the term “sample” denotes bone marrow, blood, serum or plasma.

Measuring the expression level of a gene can be performed by a variety of techniques well known in the art.

10 Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's
15 instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

20 Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments,
25 it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a
30 detectable label). A “detectable label” is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is

bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N-[3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, antl1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5''dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichloro-2-pyrimidinyl)-2'-dimethoxy-4'-dimethylaminophenylazo-3-carboxyfluorescein (DTAF), 2',7'-dimethoxy-4'-dimethylaminophenylazo-3-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein

(OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A);
5 rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine
10 isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No.
15 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6, 130, 101 and 6,716,979), the BODIPY series of dyes (dipyrrrometheneboron difluoride dyes, for example as described in U.S. Pat.
20 Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM
25 (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649, 138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap
30 of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in,

for example, Bruchez et al., Science 281 :20132016, 1998; Chan et al., Science 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927, 069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No. 2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

Additional labels include, for example, radioisotopes (such as ^3H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd^{3+} , and liposomes.

Detectable labels that can be used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in

situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g., genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and 5,427,932; and for example, in Pir1kel et al., Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al., Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al., Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al., Am. J. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH.

Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, coumarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 01 17153.

It will be appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can be labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 nm). Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct,

and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly match a nucleic acid of interest, to be amplified. The probes and primers are "specific" to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the specific sequences.

In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface. The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man

skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression level of a gene by comparing its expression to the expression of a gene that is not a relevant for determining the cancer stage of the patient, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S gene, GUSB, PGK1 and TFRC. According to the invention the housekeeping genes used were GAPDH, GUSB, TBP and ABL1. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

Predetermined reference values used for comparison may comprise “cut-off” or “threshold” values that may be determined as described herein. Each reference (“cut-off”) value for each gene of interest may be predetermined by carrying out a method comprising the steps of

a) providing a collection of samples from patients suffering of AML treated or not with traditional cytarabine and/or anthracycline;

b) determining the expression level of the gene for each sample contained in the collection provided at step a);

c) ranking the tumor tissue samples according to said expression level

d) classifying said samples in pairs of subsets of increasing, respectively decreasing, number of members ranked according to their expression level,

e) providing, for each sample provided at step a), information relating to the responsiveness of the patient or the actual clinical outcome for the corresponding cancer patient (i.e. the duration of the event-free survival (EFS), relapse free survival (RFS) or the overall survival (OS) or both);

f) for each pair of subsets of samples, obtaining a Kaplan Meier percentage of survival curve;

g) for each pair of subsets of samples calculating the statistical significance (p value) between both subsets

h) selecting as reference value for the expression level, the value of expression level for which the p value is the smallest.

For example the expression level of a gene X has been assessed for 100 AML samples of 100 patients. The 100 samples are ranked according to their expression level. Sample 1 has the best expression level and sample 100 has the worst expression level. A first grouping provides two subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding AML patient, Kaplan Meier curves are prepared for each of the 99 groups of two subsets. Also for each of the 99 groups, the p value between both subsets was calculated.

The reference value is selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the expression level corresponding to the boundary between both subsets for which the p value is minimum is considered as the reference value. It should be noted that the reference value is not necessarily the median value of expression levels.

In routine work, the reference value (cut-off value) may be used in the present method to discriminate AML samples and therefore the corresponding patients.

Kaplan–Meier curves of percentage of survival as a function of time are commonly to measure the fraction of patients living for a certain amount of time after treatment and are well known by the man skilled in the art.

The man skilled in the art also understands that the same technique of assessment of the expression level of a gene should of course be used for obtaining the reference value and thereafter for assessment of the expression level of a gene of a patient subjected to the method of the invention.

Such predetermined reference values of expression level may be determined for any gene defined above.

According to the invention, the level of the protein of the selected genes (the protein Chk1 for the gene CHK1 and the protein Pol theta for the gene POLQ) may also be measured to determine the response of a patient affected with an acute myeloid leukemia (AML) to cytarabine and/or anthracycline treatment or to determine the survival time of a patient affected with an acute myeloid leukemia (AML).

Thus, the invention also relates to a method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i) determining in a sample obtained from the patient the

concentration of the Chk1 and/or Pol theta proteins ii) comparing the concentration determined at step i) with its predetermined reference value and iii) providing a good prognosis when the concentration determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the concentration determined at step i) is higher than its predetermined reference value.

The invention also relates to a method for predicting the responsiveness of a patient affected with an acute myeloid leukemia (AML) to a cytarabine and/or anthracycline treatment comprising i) determining in a sample obtained from the patient the concentration of the Chk1 and/or Pol theta proteins ii) comparing the concentration determined at step i) with its predetermined reference value wherein when the concentration determined at step i) is lower than its predetermined reference values then the responsiveness of the patient to the treatment is good, and when the concentration determined at step i) is higher than its predetermined reference value then the responsiveness of the patient to the treatment is bad.

Measuring the level of the protein can be performed by a variety of techniques well known in the art.

Typically protein concentration may be measured for example by capillary electrophoresis-mass spectroscopy technique (CE-MS) or ELISA performed on the sample.

Detection of protein concentration in the sample may also be performed by measuring the level of proteins (Chk1Pol or theta). In the present application, the "level of proteins" means the quantity or concentration of said proteins. In another embodiment, the "level of proteins" means the level of proteins fragments (Chk1 or Pol theta fragments).

Such methods comprise contacting a sample with a binding partner capable of selectively interacting with proteins present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal.

The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, capillary electrophoresis-mass spectroscopy technique (CE-MS).etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies against the proteins to be tested. A sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule is added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate is washed and the presence of the secondary binding molecule is detected using methods well known in the art.

Methods of the invention may comprise a step consisting of comparing the proteins and fragments concentration in circulating cells with a control value. As used herein, "concentration of proteins" refers to an amount or a concentration of a transcription product, for instance the proteins Chk1 or Pol theta. Typically, a level of a protein can be expressed as nanograms per microgram of tissue or nanograms per milliliter of a culture medium, for example. Alternatively, relative units can be employed to describe a concentration. In a particular embodiment, "concentration of proteins" may refer to fragments of the proteins (Chk1 or Pol theta). Thus, in a particular embodiment, fragments of Chk1 or Pol theta may also be measured.

A further object of the invention relates to kits for performing the methods of the invention, wherein said kits comprise means for measuring the expression level of CHK1 and/or POLQ genes of the invention in the sample obtained from the patient.

The kits may include probes, primers macroarrays or microarrays as above described. For example, the kit may comprise a set of probes as above defined, usually made of DNA, and that may be pre-labelled. Alternatively, probes may be unlabelled and the ingredients for labelling may be included in the kit in separate containers. The kit may further comprise hybridization reagents or other suitably packaged reagents and materials needed for the particular hybridization protocol, including solid-phase matrices, if applicable, and standards.

Alternatively the kit of the invention may comprise amplification primers that may be pre-labelled or may contain an affinity purification or attachment moiety. The kit may further comprise amplification reagents and also other suitably packaged reagents and materials needed for the particular amplification protocol.

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Therapeutic methods

10 A second aspect of the invention relates to i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML).

15 In one embodiment, the invention relates to an inhibitor of Chk1 protein and ii) an inhibitor of Pol theta protein as a combined preparation for simultaneous for use in the treatment of acute myeloid leukemia (AML).

20 As used herein, the term “inhibitor” denotes a molecule which can inhibit the activity of the protein (e.g. inhibit the kinase or polymerase activity of the proteins) or a molecule which destabilizes the protein.

In another embodiment, the invention relates to an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression for use in the treatment of acute myeloid leukemia (AML).

25 In another embodiment, the invention relates to an inhibitor of Chk1 for use in the treatment of acute myeloid leukemia (AML).

In still another embodiment, the invention relates to an inhibitor of polQ or an inhibitor of the POLQ gene expression for use in the treatment of acute myeloid leukemia (AML).

30 Typically, the antagonist or the inhibitor according to the invention includes but is not limited to a small organic molecule, an antibody, and a polypeptide.

In one embodiment, the inhibitor according to the invention may be a low molecular weight compound, e. g. a small organic molecule (natural or not).

The term "small organic molecule" refers to a molecule (natural or not) of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 10000 Da, more preferably up to 5000 Da, more preferably up to 2000 Da and most preferably up to about 1000 Da.

In one embodiment, the inhibitor of Chk1 according to the invention may be the Chk1 inhibitor 7-hydroxystaurosporine as described in D Sampath, et al., 2005.

In one embodiment, the Chk1 inhibitor is an inhibitor described in Prudhomme, Recent Patents on Anti-Cancer Drug Discovery, 2006, 1, 55-68; Expert Opin. Ther. Patents (2011) 21(8): 1191-1210; and Cell Cycle (2011) 10: 13, 2121-2128, each of which is incorporated herein by reference.

In another embodiment, the Chk1 inhibitor is selected from the group consisting of AZD7762, LY2603618, PF-00477736, and SCH 900776 (See Cancer Res. (2010) 70(12): 4972 et seq.; Clin. Cancer Res. (2010) 16(7): 2076-2084; and Shibata et al, Cancer Sci. (2011), each of which is incorporated herein by reference).

In still another embodiment, an inhibitor of the Chk1 according to the invention may be a compound as described in the patent application WO2008132500.

In one embodiment, the compound according to the invention is an antibody. Antibodies directed against the Chk1 protein or the Pol theta protein can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against Chk1 protein or the Pol theta protein can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975); the human B-cell hybridoma technique (Cote et al., 1983); and the EBV-hybridoma technique (Cole et al. 1985). Alternatively, techniques described for the production of single chain

antibodies (see e.g., U.S. Pat. No. 4,946,778) can be adapted to produce anti-Chk1 protein, or anti-Pol theta protein single chain antibodies. Compounds useful in practicing the present invention also include anti-Chk1 protein, or anti-Pol theta protein antibody fragments including but not limited to F(ab')₂ fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to Chk1 protein or Pol theta protein.

Humanized anti-Chk1 protein, or anti-Pol theta protein antibodies and antibody fragments therefrom can also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

Then, for this invention, neutralizing antibodies of Chk1 protein or Pol theta protein are selected.

In one embodiment, the compound according to the invention is an anti-Chk1 protein antibody.

In a particular embodiment, the antibody according to the invention may be the sc-8408 antibody as sent by Santa Cruz biotechnology.

In another embodiment, the compound according to the invention is an anti-Pol theta protein antibody.

In a particular embodiment, the antibody according to the invention may be the 1C11 antibody as send by Sigma-Aldrich.

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In one embodiment, the compound according to the invention is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

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Then, for this invention, neutralizing aptamers of Chk1 protein or Pol theta protein are selected.

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In one embodiment, the compound according to the invention is a polypeptide or a peptide.

In a particular embodiment the polypeptide is a functional equivalent of Chk1 or Pol theta proteins. As used herein, a "functional equivalent" of Chk1 or Pol theta is a compound which is capable of binding to Chk1 or Pol Theta, thereby preventing the interaction of Chk1 or Pol theta with their molecular partners. The term "functional equivalent" includes fragments and mutants of Chk1 or Pol theta. The term "functionally equivalent" thus includes any equivalent of Chk1 or Pol theta obtained by altering the amino acid sequence, for example by one or more amino acid deletions, substitutions or additions such that the protein analogue retains the ability to bind to these proteins. Amino acid substitutions may be made, for example, by point mutation of the DNA encoding the amino acid sequence.

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The polypeptides of the invention may be produced by any suitable means, as will be apparent to those of skill in the art. In order to produce sufficient amounts of Pol Theta or Chk1 proteins or functional equivalents thereof for use in accordance with the present

invention, expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the polypeptide of the invention. Preferably, the polypeptide is produced by recombinant means, by expression from an encoding nucleic acid molecule. Systems for cloning and expression of a polypeptide in a variety of different host cells are well known.

When expressed in recombinant form, the polypeptide is preferably generated by expression from an encoding nucleic acid in a host cell. Any host cell may be used, depending upon the individual requirements of a particular system. Suitable host cells include bacteria mammalian cells, plant cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells. HeLa cells, baby hamster kidney cells and many others. Bacteria are also preferred hosts for the production of recombinant protein, due to the ease with which bacteria may be manipulated and grown. A common, preferred bacterial host is E coli.

In specific embodiments, it is contemplated that polypeptides used in the therapeutic methods of the present invention may be modified in order to improve their therapeutic efficacy. Such modification of therapeutic compounds may be used to decrease toxicity, increase circulatory time, or modify biodistribution. For example, the toxicity of potentially important therapeutic compounds can be decreased significantly by combination with a variety of drug carrier vehicles that modify biodistribution. In example adding dipeptides can improve the penetration of a circulating agent in the eye through the blood retinal barrier by using endogenous transporters.

A strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers; and modify the rate of clearance from the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

Polyethylene glycol (PEG) has been widely used as a drug carrier, given its high degree of biocompatibility and ease of modification. Attachment to various drugs, proteins, and liposomes has been shown to improve residence time and decrease toxicity. PEG can be coupled to active agents through the hydroxyl groups at the ends of the chain and via other chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids were explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would

have the added advantage of numerous attachment points per molecule (providing greater drug loading), and which could be synthetically designed to suit a variety of applications.

Those of skill in the art are aware of PEGylation techniques for the effective modification of drugs. For example, drug delivery polymers that consist of alternating polymers of PEG and tri-functional monomers such as lysine have been used by VectraMed (Plainsboro, N.J.). The PEG chains (typically 2000 daltons or less) are linked to the α - and ϵ -amino groups of lysine through stable urethane linkages. Such copolymers retain the desirable properties of PEG, while providing reactive pendent groups (the carboxylic acid groups of lysine) at strictly controlled and predetermined intervals along the polymer chain. The reactive pendent groups can be used for derivatization, cross-linking, or conjugation with other molecules. These polymers are useful in producing stable, long-circulating pro-drugs by varying the molecular weight of the polymer, the molecular weight of the PEG segments, and the cleavable linkage between the drug and the polymer. The molecular weight of the PEG segments affects the spacing of the drug/linking group complex and the amount of drug per molecular weight of conjugate (smaller PEG segments provides greater drug loading). In general, increasing the overall molecular weight of the block co-polymer conjugate will increase the circulatory half-life of the conjugate. Nevertheless, the conjugate must either be readily degradable or have a molecular weight below the threshold-limiting glomerular filtration (e.g., less than 60 kDa).

In addition, to the polymer backbone being important in maintaining circulatory half-life, and biodistribution, linkers may be used to maintain the therapeutic agent in a pro-drug form until released from the backbone polymer by a specific trigger, typically enzyme activity in the targeted tissue. For example, this type of tissue activated drug delivery is particularly useful where delivery to a specific site of biodistribution is required and the therapeutic agent is released at or near the site of pathology. Linking group libraries for use in activated drug delivery are known to those of skill in the art and may be based on enzyme kinetics, prevalence of active enzyme, and cleavage specificity of the selected disease-specific enzymes. Such linkers may be used in modifying the protein or fragment of the protein described herein for therapeutic delivery.

In another embodiment, the compound according to the invention is an inhibitor of CHK1 or POLQ gene expression.

Small inhibitory RNAs (siRNAs) can also function as inhibitors of CHK1 or POLQ expression for use in the present invention. CHK1 or POLQ gene expression can be reduced

by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that CHK1 or POLQ gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see for example Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of CHK1 or POLQ gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of CHK1 or POLQ mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of CHK1 or POLQ gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells and preferably cells expressing CHK1 or POLQ. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the the antisense oligonucleotide siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing

multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in
5 tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al.,
10 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322,
15 pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, eye, intradermal, subcutaneous, or other routes. It
20 may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a particular embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter may be specific for Muller glial cells, microglia cells, endothelial cells, pericyte cells and astrocytes. For example, a specific expression in Muller glial cells may be obtained through the promoter of the glutamine synthetase gene is suitable.
25 The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

Another object of the invention relates to a method for treating AML comprising administering to a subject in need thereof a therapeutically effective amount of i) an inhibitor

of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the PolQ gene expression as defined above.

In another embodiment, the invention relates to a method for treating AML comprising administering simultaneously, separately or sequentially to a subject in need thereof a therapeutically effective amount of i) an inhibitor of Chk1 or an inhibitor of the
5 CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the PolQ gene expression as defined above.

Another object of the invention relates to a method for treating AML comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of
10 Chk1 or an inhibitor of the CHK1 gene expression as defined above.

Another object of the invention relates to a method for treating AML comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of Pol theta or an inhibitor of the PolQ gene expression as defined above.

15 In another object, the invention relates to i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as described above.

20 In another object, the invention relates to i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML) in patient with a bad response to a cytarabine and/or anthracycline treatment as described above.

25 According to the invention, patients with a higher expression level for POLQ and/or CHK1 than their predetermined reference values as described above are eligible for a treatment using an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and/or an inhibitor of Pol theta or an inhibitor of the POLQ gene expression according to the invention.

30 Thus, the methods of the invention as described above may be useful to determine the eligibility of a patient to be treated with inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and/or an inhibitor of Pol theta or an inhibitor of the POLQ gene expression.

According to the invention, the methods of the invention may be used as a companion diagnostic under a treatment of patient affected with AML.

In another embodiment, the invention relates to i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as described above.

In one embodiment, the invention relates to i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as described above.

In another embodiment, the invention relates to i) an inhibitor of Chk1 for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as described above.

Therapeutic composition

A third object of the invention relates to a pharmaceutical composition comprising i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression for use in the treatment of acute myeloid leukemia (AML).

A third object of the invention relates to a pharmaceutical composition comprising an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression for use in the treatment of acute myeloid leukemia (AML).

Any therapeutic agent of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

5 The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, parenteral, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

10 Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

15 The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

In addition, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently can be used.

20 Pharmaceutical compositions of the present invention may comprise a further therapeutic active agent. The present invention also relates to a kit comprising a compound according to the invention and a further therapeutic active agent.

For example, anti-cancer agents may be added to the pharmaceutical composition as described below.

25 Anti-cancer agents may be for example cytarabine, anthracyclines, fludarabine, gemcitabine, capecitabine, methotrexate, taxol, taxotere, mercaptopurine, thioguanine, hydroxyurea, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, etoposide, teniposide, campathecins, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epimbicm, 5-fluorouracil, taxanes
30 such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, nitrogen mustards, BCNU, nitrosoureas such as carmustine and lomustine, vinca alkaloids such as vinblastine, vincristine and vinorelbine, imatinib mesylate, hexamethylnelamine, topotecan, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrosine kinase inhibitors, protease inhibitors, inhibitors herbimycin A, genistein, erlotinib, and lapatinib. In one

embodiment, additional anticancer agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates, pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxin, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycins, bleomycins, MDR inhibitors and Ca^{2+} ATPase inhibitors.

Additional anti-cancer agents may be selected from, but are not limited to, cytokines, chemokines, growth factors, growth inhibitory factors, hormones, soluble receptors, decoy receptors, monoclonal or polyclonal antibodies, mono-specific, bi-specific or multi-specific antibodies, monobodies, polybodies.

Additional anti-cancer agent may be selected from, but are not limited to, growth or hematopoietic factors such as erythropoietin and thrombopoietin, and growth factor mimetics thereof.

In the present methods for treating cancer the further therapeutic active agent can be an antiemetic agent. Suitable antiemetic agents include, but are not limited to, metoclopramide, domperidone, prochlorperazine, promethazine, chlorpromazine, trimethobenzamide, ondansetron, granisetron, hydroxyzine, acethylleucine monoemanolamine, alizapride, azasetron, benzquinamide, bietanautine, bromopride, buclizine, clebopride, cyclizine, dunenhydrinate, diphenidol, dolasetron, meclizme, methallatal, metopimazine, nabilone, oxypemdy, pipamazine, scopolamine, sulpiride, tetrahydrocannabinols, thiefhyperazine, thioproperazine and tropisetron. In a preferred embodiment, the antiemetic agent is granisetron or ondansetron.

In another embodiment, the further therapeutic active agent can be an hematopoietic colony stimulating factor. Suitable hematopoietic colony stimulating factors include, but are not limited to, filgrastim, sargramostim, molgramostim and epoietin alpha.

In still another embodiment, the other therapeutic active agent can be an opioid or non-opioid analgesic agent. Suitable opioid analgesic agents include, but are not limited to, morphine, heroin, hydromorphone, hydrocodone, oxymorphone, oxycodone, metopon, apomorphine, nomioipine, etoipbine, buprenorphine, mepeddine, lopermide, anileddine, ethoheptazine, piminidine, betaprodine, diphenoxylate, fentanil, sufentanil, alfentanil, remifentanil, levorphanol, dextromethorphan, phenazodone, pemazocine, cyclazocine, methadone, isomethadone and propoxyphene. Suitable non-opioid analgesic agents include, but are not limited to, aspirin, celecoxib, rofecoxib, diclofinac, diflusal, etodolac,

fenoprofen, flurbiprofen, ibuprofen, ketoprofen, indomethacin, ketorolac, meclofenamate, mefanamic acid, nabumetone, naproxen, piroxicam and sulindac.

In yet another embodiment, the further therapeutic active agent can be an anxiolytic agent. Suitable anxiolytic agents include, but are not limited to, buspirone, and
5 benzodiazepines such as diazepam, lorazepam, oxazepam, chlorazepate, clonazepam, chlordiazepoxide and alprazolam.

Screening method

10 In a further aspect, the present invention relates to a method of screening a candidate compound for use in the treatment of acute myeloid leukemia (AML), wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that inhibit Chk1 or CHK1 gene expression or inhibit Pol theta or POLQ gene
15 expression.

In a further aspect, the present invention relates to a method of screening a candidate compound for use in the treatment of acute myeloid leukemia (AML) in a subject in need thereof, wherein the method comprises the steps of:

- 20 - providing a cell, tissue sample or organism expressing the Chk1/CHK1 or Pol theta/POLQ,
- providing a candidate compound such as small organic molecule, antibodies, peptide or polypeptide,
- measuring the activity of the Chk1/CHK1 or Pol theta/POLQ,
- 25 - and selecting positively candidate compounds that inhibit Chk1/CHK1 or Pol theta/POLQ.

Methods for measuring the inhibition of Chk1/CHK1 or Pol theta/POLQ are well known in the art. For example, monitoring in cellulo the defective checkpoint function of
30 Chk1 by measuring the lack of phosphorylation of Chk1 or its substrates, such Cdc25, upon replication stress (Hydroxyurea, MMS, UV) and monitoring in vitro the defective DNA synthesis by Pol theta by measuring decreased dNTPs incorporation in a primer extension assay may be done.

Tests and assays for screening and determining whether a candidate compound is a Chk1/CHK1 or Pol theta/POLQ inhibitor are well known in the art. In vitro and in vivo assays may be used to assess the potency and selectivity of the candidate compounds to inhibit Chk1/CHK1 or Pol theta/POLQ.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Overall survival (A; $p = 0.005$), event free survival (B; $p = 0.002$) and relapse free survival (C; $p = 0.002$) in percentage according to CHK1 expression. Analyse time in months.

Figure 2: Overall survival (A; $p = 0.02$), event free survival (B; $p = 0.01$) and relapse free survival (C; $p = 0.06$) in percentage according to POLQ expression. Analyse time in months.

Figure 3: Overall survival (A; $p = 0.02$), event free survival (B; $p = 0.01$) and relapse free survival (C; $p = 0.02$) in percentage according to the combination CHK1/POLQ expression. Analyse time in months.

Figure 4: High Chk1 level favors resistance to Ara-C treatment, and CHK1 inhibition restores sensitivity to Ara-C.

AML blast cells were grown in clonogenic assays in continuous exposure to 5 or 10 nM Ara-C alone, or in combination with 250 nM SCH900776. Colony formation was assessed after 7 days and represented as the ratio of the number of clones scored between untreated and treated condition. Horizontal lines correspond to mean value ($n=22$ for Low Chk1 expressing cells (L) ; $n=15$ for High Chk1 expressing cells (H)). Statistical analyses were performed using a Mann Whitney test (5 and 10 nM Ara-C, *, $p=0.0242$ and $p= 0.0173$ respectively, and 10 nM Ara-C - 250nM SCH900776, *, $p=0.0398$).

EXAMPLES:**Material & Methods***Patients*

Between Jan 1, 2000, and Dec 31, 2010, 513 consecutive patients (65 years of age or younger) with a new diagnosis of AML have been treated by intensive chemotherapy in our center. Diagnosis workup and treatment modalities have been described elsewhere (Bertoli S. Blood. 2013;121(14):2618-26. LaRoche O. Oncotarget. 2011;2(11):850-61.). The cytogenetic risk was established according to the Medical Research Council classification (Grimwade D, Blood, 2010). In this cohort of patients, 198 samples stored after informed consent in the HIMIP tumor bank of the U1037 Inserm department (n°DC-2008-307-CPTP1 HIMIP) were available for this study. The characteristics and outcome of the remaining 315 non tested patients as compared to the 198 tested patients are shown in supplementary table 1. This study was approved by the institutional review board (Ethical Committee of Research).

Total RNA extraction

Total RNA were extracted from frozen cells (7 to 15 million of cells) stored in 1ml of Tri Reagent RNA/DNA/protein isolation reagent (Molecular Research Center). The extraction was done by adding 200µl of cold Ready Red-Chloroform isoamyl alcohol (MP Biomedicals, France) and vigorously shaken for 15 s using a vortex, then incubated on ice during at least 5 min. After centrifugation at 13 000 rpm for 15min at 4°C, the upper aqueous phase was transferred into a new tube. One volume of isopropanol was added, vortexed and incubated for one hour at -20°C. After centrifugation at 13 000 rpm for 15min at 4°C, the pellet was dried and 1ml of cold 75% ethanol added. After centrifugation at 8000 rpm for 10min at 4°C, the pellet was dried and incubated during 4 min at 65°C. Then, 30µl of RNase free water was added to the pellet with RiboLock RNase Inhibitor (40U, Fermentas, France) and mixed. RNA concentration will be determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Thermo Scientific, Brebières, France).

Quality Control of RNA

RNA quality and purity was assessed on the Agilent 2100 BioAnalyser by using the Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). Only RNA

presenting a RIN>6.5 were selected for expression analysis (Around 90% of the samples have a RIN>8).

Reverse transcription-PCR

5 cDNA was generated from 1µg of RNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen) for reverse transcription-PCR following the manufacturer's suggestions. In order to ensure good quality reverse transcription step, one part of each cDNA was used to check ABL1 ("TaqMan Gene Expression Assay", Applied Biosystems, Hs01104728_m1) expression level homogeneity using ABI Prism 7300 HT (Applied Biosystems, Carlsbad, CA, 10 USA).

Specific target amplification

The other part of each cDNA was diluted in water to 5ng/µl and dedicated to target amplification for Biomark Dynamic Arrays (Fluidigm, BioMark, Pueblo, CO, USA). 15 Inventoried TaqMan assays (Applied Biosystems) were pooled using 84 probes and primers pairs (72 DNA replication genes, 9 housekeeping genes and 3 genes, BAALC, ERG, and MN1, whose expression was reported to negatively impact on outcome of cytogenetically normal AML patients), to a final concentration of 0,2X for each of the 84 assays. To increase sensitivity, a multiplexed preamplification process was performed for the pool on every 1,25 20 µl cDNA using 14 cycles cDNA preamplification step (95°C for 15s and 60°C 4 min) and Taqman PreAmp Master Mix (Applied Biosystems) in a standard PCR Thermocycler.

Quantitative PCR

Preamplified cDNA was diluted 1:5 in 10 mmol/L Tris, 1 mmol/L EDTA. Diluted 25 cDNA (2.25 µl) was added to 2.5 µl Taqman Universal PCR Master Mix (Applied Biosystems) and 0.25 µl GE Sample Loading Reagent (Fluidigm). In a separate tube, 3.5 µl of Taqman Assay was added to 3.5 µl Sample Loading Reagent. 5 µl cDNA samples were loaded into the sample inlet wells, and 5 µl assay samples were loaded into assay detector inlets.

30 Because 198 samples were to be analyzed in duplicated, five 96.96 Dynamic Arrays (Fluidigm) were used. For each plate, 1 well was loaded with H2O as control for contamination. Genomic DNA (gDNA) from 3 different patients were loaded in order to check if TaqMan assays can also amplify genomic DNA. To verify STA efficiency, a sample control gDNA and assay control RNase P Taqman probe was treated (Lifetech PN 4316844),

preamplified and quantified using the same mastermix. The expected value cq was between 12 and 13. In order to do inter-plate calibration, a sample calibrator made of cDNA from the patient #1 was included in duplicate in each plate. The chip was primed and placed into the NanoFlex Integrated fluidic circuit controller where 8 nl of cDNA and 1 nl of Assay were mixed. Real time PCR analysis was completed on the BioMark System (Fluidigm).

Data processing

Raw data obtained from the system's software using the auto detector function in order to establish the threshold setting (BioMark Realtime PCR Analysis V2.1.1, Fluidigm), were checked using the graphical representation of the plate layout. Among all reactions investigated, 0 were rejected due to bubbles or instable ROX signal. All amplification curves were displayed for each well of the calibrator sample. When threshold for cycle did not meet quality criteria (i.e. threshold set in the linear phase of the amplification curve instead of the exponential phase), threshold value was set manually. The threshold established for the first Dynamic Array were applied to the 4 other Dynamic Arrays. Wells with very high (>26), absent (999) or very low (<2) endogenous Ct resulted in exclusion.

Normalization method

In order to perform the real time qPCR data normalization with the housekeeping genes and the inter-plate calibration have been performed by using the qbase+ algorithm as described 14. Among the 9 housekeeping genes tested (GUSB (Hs99999908_m1), ACTB (Hs99999903_m1), ABL1 (Hs01104728_m1), G6PD (Hs00166169_m1), TBP (Hs00427621_m1), GAPDH (Hs03929097_g1), HMBS (Hs00609293_g1), B2M (Hs00984230_m1), UBC (Hs00824723_m1)), GeNorm algorithm determined the four most stable which were GAPDH, GUSB, TBP and ABL1 and calculate the gene expression normalization factor. Expression values are given in $\Delta\Delta Ct$. BAALC (Hs00227249_m1), ERG (Hs01554635_m1) and MN1 (Hs00159202_m1) genes, whose the expressions were previously described to be correlated with the prognosis of patients with CN-AML5-7, were used in order to validate our data.

DNA context sequences and applied biosystems references

Assay ID	Gene
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Hs00967506_m1	CHEK1
Hs00198196_m1	POLQ

Statistical analysis

We explored the association between the expression of 72 DNA replication genes of interest and the different survival endpoints. Complete response (CR) required a normocellular bone marrow with less than 5% blasts and no Auer rods, neutrophil count $\geq 1 \times 10^9/L$ and platelet count $\geq 100 \times 10^9/L$, without evidence of extramedullary disease after one or two courses of treatment. Induction failures included deaths in aplasia and resistant disease. Overall survival (OS) was calculated from the date of the first day of chemotherapy until the date of death from any cause. Event-free survival (EFS) was calculated from the first day of chemotherapy until the date of treatment failure, relapse, or patient death from any cause. Relapse-free survival (RFS) for patients who achieved CR was calculated from the date of CR until the date of relapse or death from any cause with censoring of other patients at the date of last follow-up. Time to relapse was evaluated as cumulative incidence of relapse (CIR) and cumulative cause-specific hazards of relapse (CSH) measured from CR date until date of relapse except for patients deceased without relapse where death was considered as competitive risk. CHK1 and POLQ expression appeared to be the most significantly associated with OS, EFS and RFS with a stable dose-effect relationship. Consequently, we focused the analysis on CHK1 and POLQ expression only. We categorized gene expression in a binary variable “high expression” versus “low expression” from the median expression value. Clinical characteristics have been compared according to the gene expression level using chi-square tests. We estimated the OS, EFS and RFS functions using the Kaplan Meier method. The median follow-up among patients who were still alive at the date of last contact (n=80) was 68.3 months (range, 29.0 to 132.0). High and low CHK1 or POLQ expression groups were compared using Log-rank tests. For multivariate analyses, we applied Cox proportional hazards models adjusted for sex, age, white blood cells count, year of diagnosis and cytogenetic risk group. An internal validation procedure has been computed in order to correct the expected over-optimism of Cox models. We applied the bootstrap cross-validation procedure: we derived slope indices from 200 bootstrap samples and used them as shrinkage factors by multiplying slope indices with regression coefficients. Slope indices have been computed using the “rms” package for R (Harrell FE [2009]: rms: S functions for

biostatistical/epidemiologic modeling, testing, estimation, validation, graphics, and prediction. Programs available from biostat.mc.vanderbilt.edu/rms). We performed the competitive risk analysis following the approach recommended by Latouche et al., using the Cox model (to calculate cause specific hazard ratios) and the Fine-Gray model (to calculate subdistribution hazard ratios), and presenting the results for all causes side by side. All analyses were performed using Stata Statistical Software (release 11.2; Stata Corporation, College Station, TX), except for the internal validation procedure that was achieved with the R (v3.0.1) (R Core Team 2013, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>).

EXAMPLE 1: CHK1 gene

Results

Characteristics of patients and response to induction chemotherapy according to CHK1 expression

We have monitored the expression of a subset of genes involved in different aspects of DNA replication, including stalled DNA replication fork stability and replication checkpoint. Among all the replication genes tested, the replication checkpoint gene CHK1 appeared to be the strongest factor associated with outcome. Thus, patients were dichotomized according to CHK1 median expression into low and high expressers. There were no significant differences between patients with low and high CHK1 expression in age, sex, white blood cell count (WBC), cytogenetics or FLT3-ITD/NPM1 status. However, there was a significant association between CHK1 expression and AML status (de novo vs. secondary). The CR rate did not differ according to CHK1 status, with 83/99 (51.2%) and 79/99 (48.8%) achieving CR in the CHK1high group and CHK1low group, respectively (p=0.46).

Impact of CHK1 expression on survival endpoints

Since high levels of BAALC, ERG, and MN1 expression were previously reported as poor prognosis markers in CN-AML patients, we first confirmed that high expression levels of these three genes measured by the Fluidigm system had a significant impact on OS in our cohort. For the 90 CN-AML patients, high expression (> median expression value) of BAALC, ERG and MN1 was associated with an increase in death rate with respective hazard ratios (HR) of 2.11 (95% CI=1.19, 3.74), 2.3 (95% CI=1.32, 4.00) and 1.63 (95% CI=0.86,

3.07) . High CHK1 expression predicted shorter EFS with 5-year estimates of 24.2% (95% CI: 16.3%-33.0%) for the CHK1high group and 50.3% (95% CI: 40.1%-59.7%) for the CHK1low group (HR=1.72, 95% CI=1.20-2.45, p=0.002) (Figure 1). For the 172 CR patients, 5-year RFS was 30.4% (95% CI:20.7%-40.7%) in the CHK1high group and 60% (95% CI: 48.6%-69.7%) in the CHK1low group (HR 1.94, 95% CI, 1.27-2.96, p=0.002) (Figure 1). The difference between both groups was also significant in 5-y CSH and CIR while there was no difference in the rate of death without relapse . OS differed significantly, with a 5-year estimate of 29.5% (95% CI: 20.7%-38.8%) in the CHK1high group and 52.4% (95% CI: 42.1%-61.7%) in the CHK1low group (HR=1.68, 95% CI=1.16-2.43, p=0.005) (Figure 1).

The multivariate analyses based on Cox proportional hazard models adjusted for age, sex, year of diagnosis, white blood cells count, and cytogenetic risk group were performed. After correction for over-optimism using the cross-validation procedure, the hazard ratios of patients with high versus low CHK1 expression were 1.58 (95% CI, 1.15 to 2.17) for OS, 1.58 (95% CI, 1.17 to 2.14) for EFS, and 1.74 (95% CI, 1.25 to 2.43) for RFS.

In subgroups analysis, CHK1 expression had no impact on OS in the good-risk group including patients with core binding factor-AML or patients with NPM1 mutation without FLT3 mutation . However, in the 57 patients who were allografted, CHK1 expression retained a significant impact on OS .

EXAMPLE 2: PolQ gene

Results

In a series of 195 patients (CHU Toulouse) with AML all treated with intensive cytarabine/anthracycline-based first-line therapy, overall survival, event free survival and relapse free survival with high expression of POLQ in peripheral lymphocytes, was significantly shorter compared with patients with low POLQ expression (Figure 2). The corresponding overall, event free and relapse free survival times estimated by the Kaplan-Meier method and results of log-rank tests are detailed in Figure 2. To assess whether POLQ expression provide additional prognostic value in the context of other clinical and molecular prognosticators, we constructed a Cox multivariate regression model to examine in all patients the effect of age, gender, cytogenetic risk group, and type of karyotype on the correlation between overall survival, event free survival, or relapse free survival and gene expression. This analysis revealed that a strong correlation was still observed after adjustment to covariates for the POL Q gene and that high POLQ expression was unfavorable prognostic

factors in multivariable analysis. A cause specific analysis of relapse using a Cox model (cause specific hazard of relapse versus death) and a Fine-Gray model (for cumulative incidence of relapse versus death) showed that high expression of POLQ seemed associated with higher rates of relapse but was not associated with higher rates of death.

EXAMPLE 3: CHK1 gene and PolQ gene

Results

To assess whether the combination CHK1/POLQ expression provide additional prognostic value in the context of other clinical and molecular prognosticators, we constructed a Cox multivariate regression model to examine in all patients the effect of age, gender, cytogenetic risk group, and type of karyotype on the correlation between overall survival, event free survival, or relapse free survival and gene expression. This analysis revealed that overall survival, event free survival and relapse free survival with high expression of the combination CHK1/POLQ in bone marrow, was significantly shorter compared with patients with low CHK1/POLQ expression (Figure 3). Compared to a combined low expression of CHK1 and POLQ, a combined high expression of CHK1 and POLQ was associated with an increase in death rate, (death or treatment failure or relapse) rate, and (death or relapse) rate with respective hazard ratios of 2.35 (95% CI=1.29-4.28), 2.27 (95%CI=1.27-4.06) and 3.29 (95%CI=1.68-6.44).

A cause specific analysis of relapse using a Cox model (cause specific hazard of relapse versus death) and a Fine-Gray model (for cumulative incidence of relapse versus death) showed that high expression of the combination ofCHK1/ POLQ seemed associated with higher rates of relapse but was not associated with higher rates of death.

EXAMPLE 4: Resistance to cytarabine in AML correlates with high Chk1 expression and is abolished by the Chk1 inhibitor SCH900776

Low- and high- Chk1 expressing AML primary cells were purified as described in the method section. They were next assessed for their ability to form colonies in methylcellulose when exposed to clinical relevant concentrations (5 and 10 nM) of cytarabine. As shown in Fig. 3, we unveiled that high Chk1 expressing cells were significantly more resistant to cytarabine compared to low CHK1 expressing cells (p=0.0242 and p=0.0173 respectively).

Since high Chk1 level correlated with higher AML cell survival in the presence of cytarabine, we then tested whether inhibition of Chk1 could restore cell sensitivity of these cells in a clonogeny assay. As shown in Fig. 3, addition of the Chk1 inhibitor SCH900776 to 10 nM cytarabine improved cytarabine sensitivity of the high-Chk1 expressing cells, which became as sensitive as the low-Chk1 expressing cells upon cytarabine alone. These data support a direct link between a high level of Chk1 and resistance to cytarabine in AML cells.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for predicting the survival time of a patient suffering from acute myeloid leukemia (AML) and treated with cytarabine and/or anthracycline comprising i)
5 determining in a sample obtained from the patient the gene expression level of at least 1 gene selected from the group consisting of CHK1 and POLQ ii) comparing the gene expression level determined at step i) with its predetermined reference value and iii) providing a good prognosis when the expression level determined at step i) is lower than its predetermined reference value, or providing a bad prognosis when the
10 expression level determined at step i) is higher than its predetermined reference value.
2. A method according to the claim 1 wherein the level of the gene CHK1 is determined
3. A method according to the claim 1 wherein the level of the gene POLQ is determined
4. An i) inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression, as a combined
15 preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML).
5. The combined preparation for use according to claim 4 wherein an inhibitor of Chk1 protein and ii) an inhibitor of Pol theta protein are used simultaneously in a combined preparation for use in the treatment of acute myeloid leukemia (AML).
- 20 6. An inhibitor of Chk1 or an inhibitor of the CHK1 gene expression for use in the treatment of acute myeloid leukemia (AML).
7. The combined preparation for use according to claims 4 or 5 or the inhibitor of Chk1 according to claim 6 wherein the inhibitor is AZD7762, LY2603618, PF-00477736, or SCH 900776.
- 25 8. A method for treating AML comprising administering to a subject in need thereof a therapeutically effective amount of i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the PolQ gene expression as defined above.

9. An i) inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ expression, as a combined preparation for simultaneous, separate or sequential for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as determined in claims 1 to 3.

10. An inhibitor of Chk1 or an inhibitor of the CHK1 gene expression for use in the treatment of acute myeloid leukemia (AML) in patient with a bad prognosis as determined in claims 1 to 3.

11. A pharmaceutical composition comprising i) an inhibitor of Chk1 or an inhibitor of the CHK1 gene expression, and ii) an inhibitor of Pol theta or an inhibitor of the POLQ gene expression for use in the treatment of acute myeloid leukemia (AML).

12. A method of screening a candidate compound for use in the treatment of acute myeloid leukemia (AML), wherein the method comprises the steps of: i) providing candidate compounds and ii) selecting candidate compounds that inhibit Chk1 or CHK1 gene expression or inhibit Pol theta or POLQ gene expression.

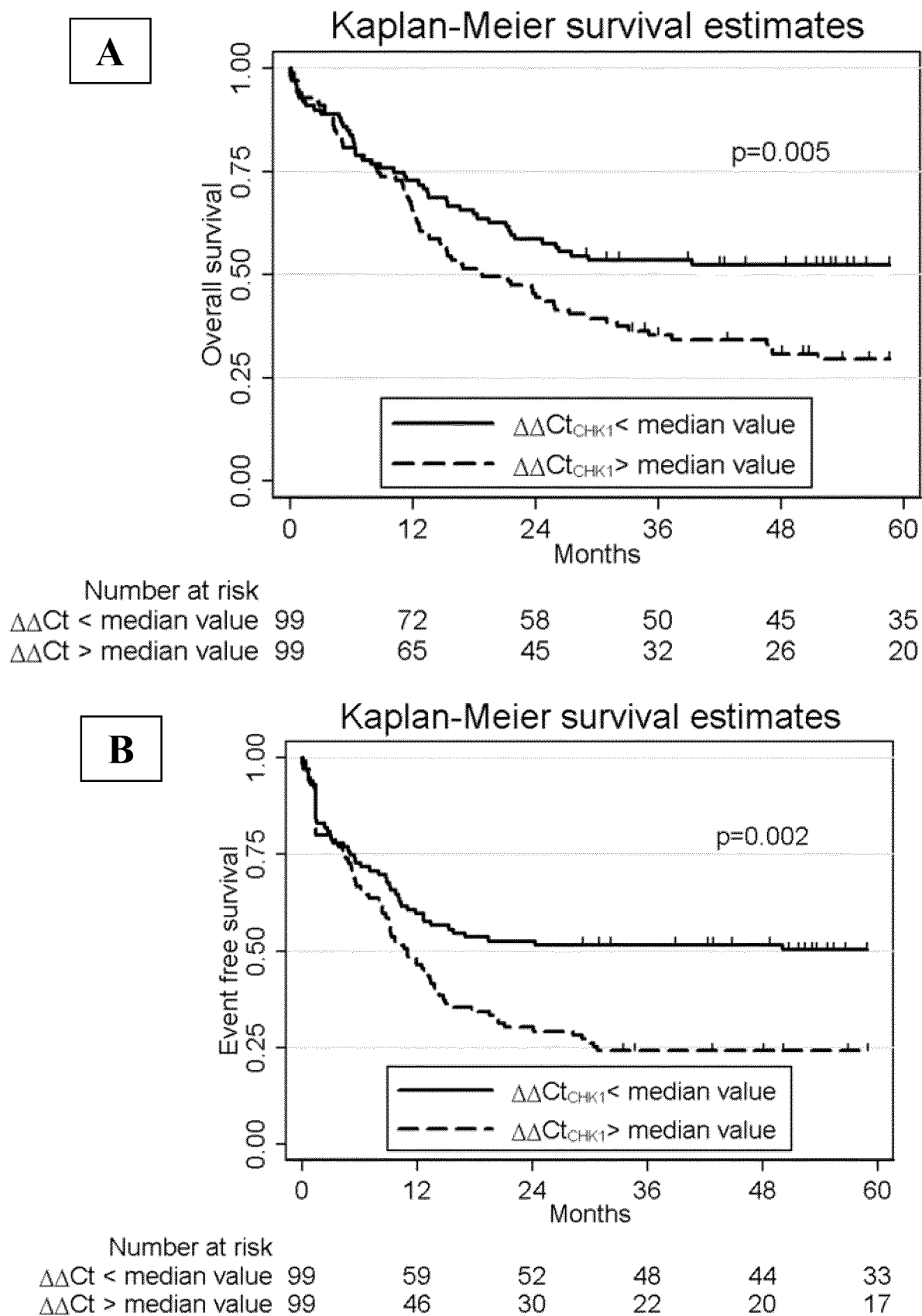


Figure 1 A and B

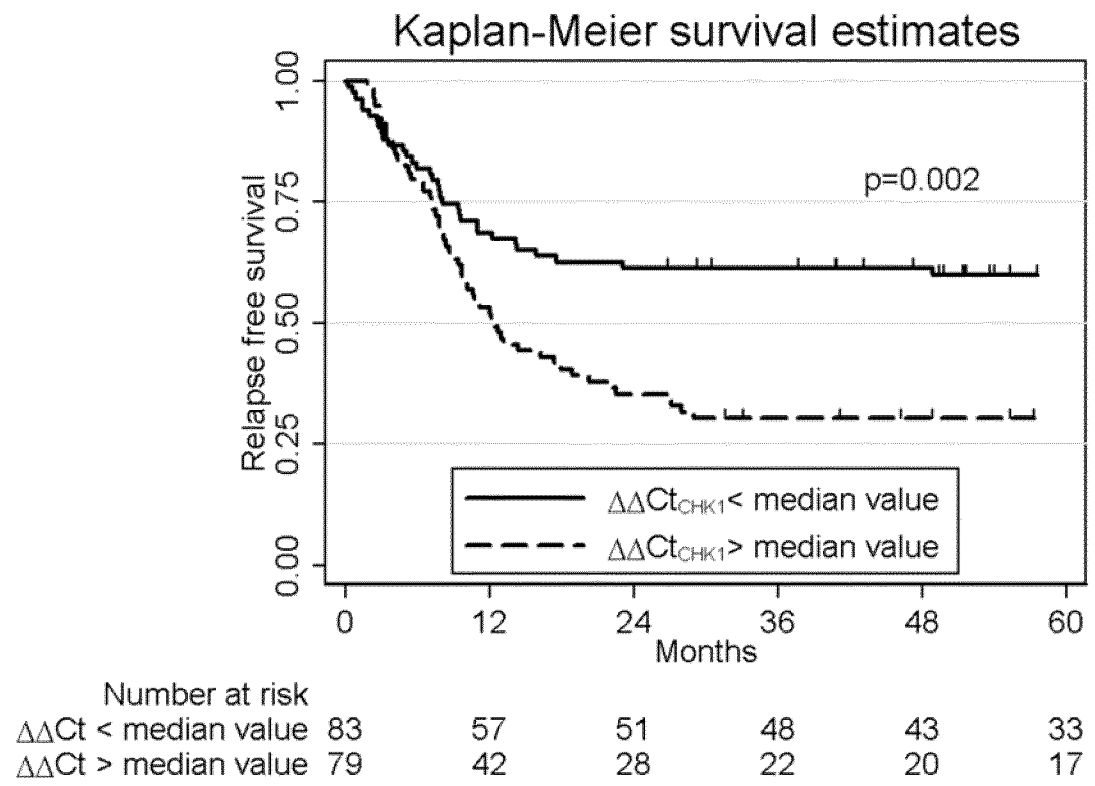


Figure 1 C

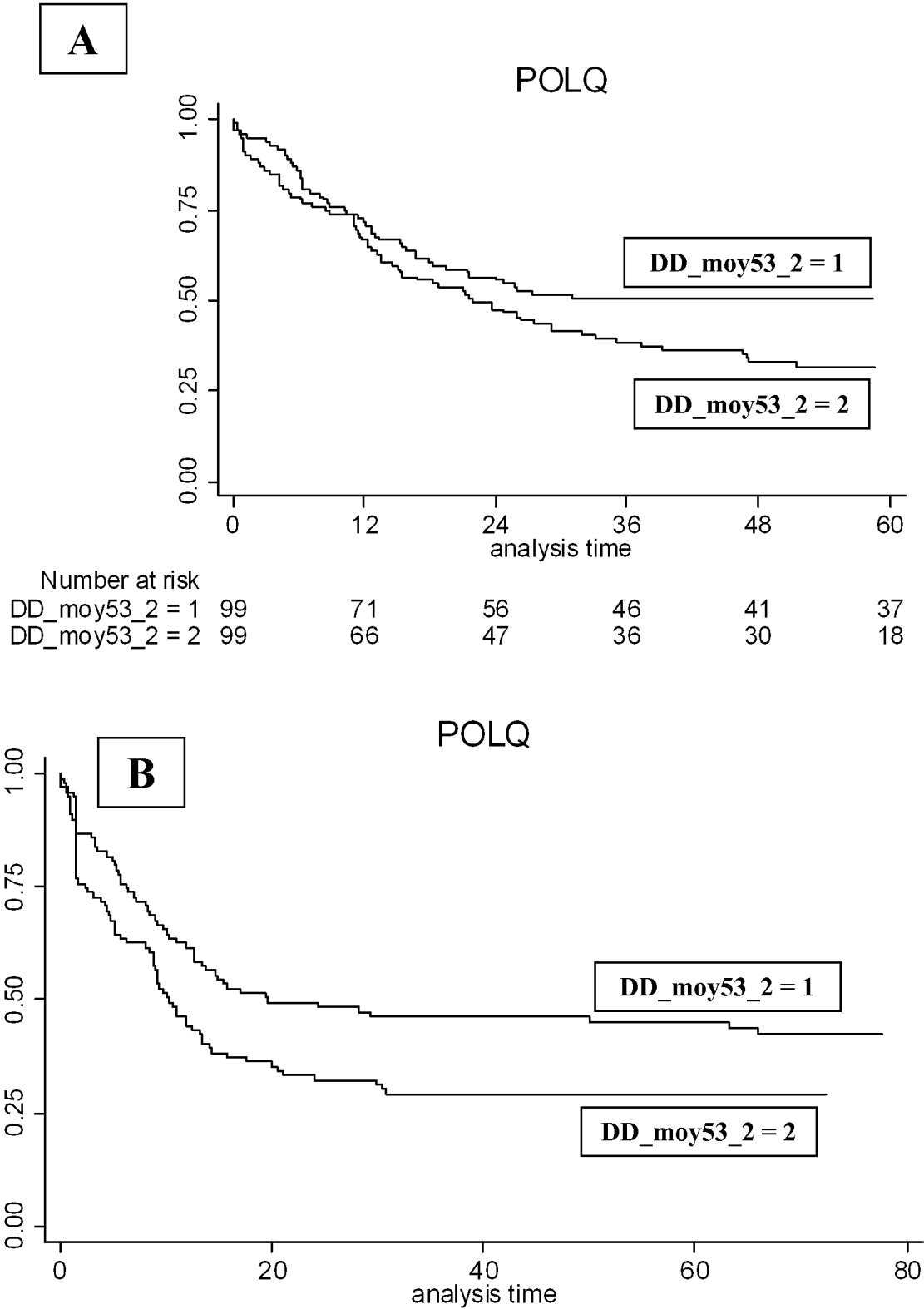


Figure 2 A and B

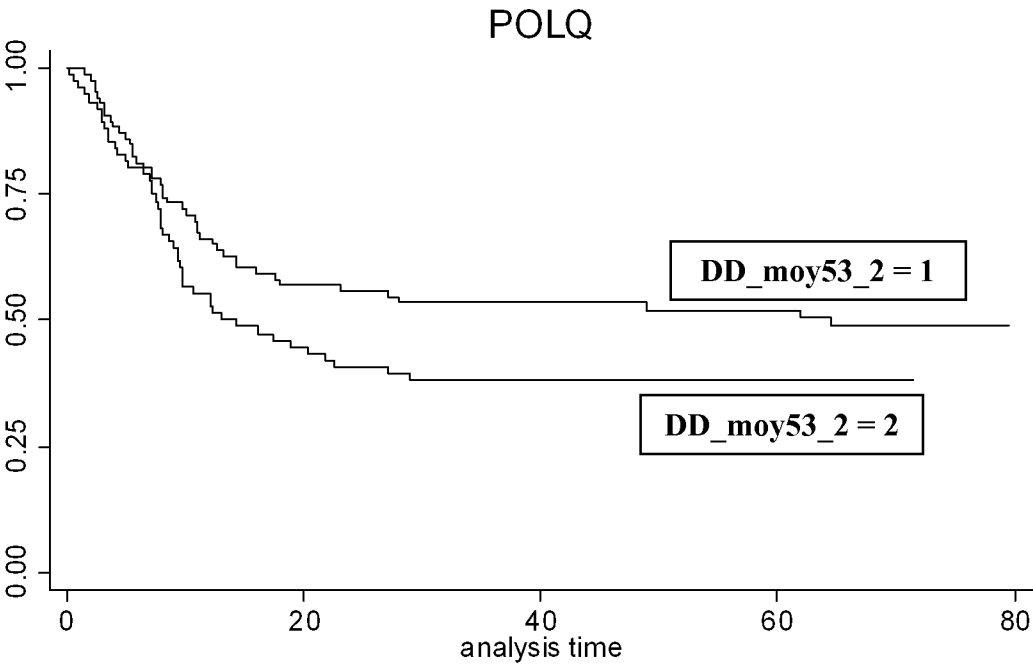


Figure 2 C

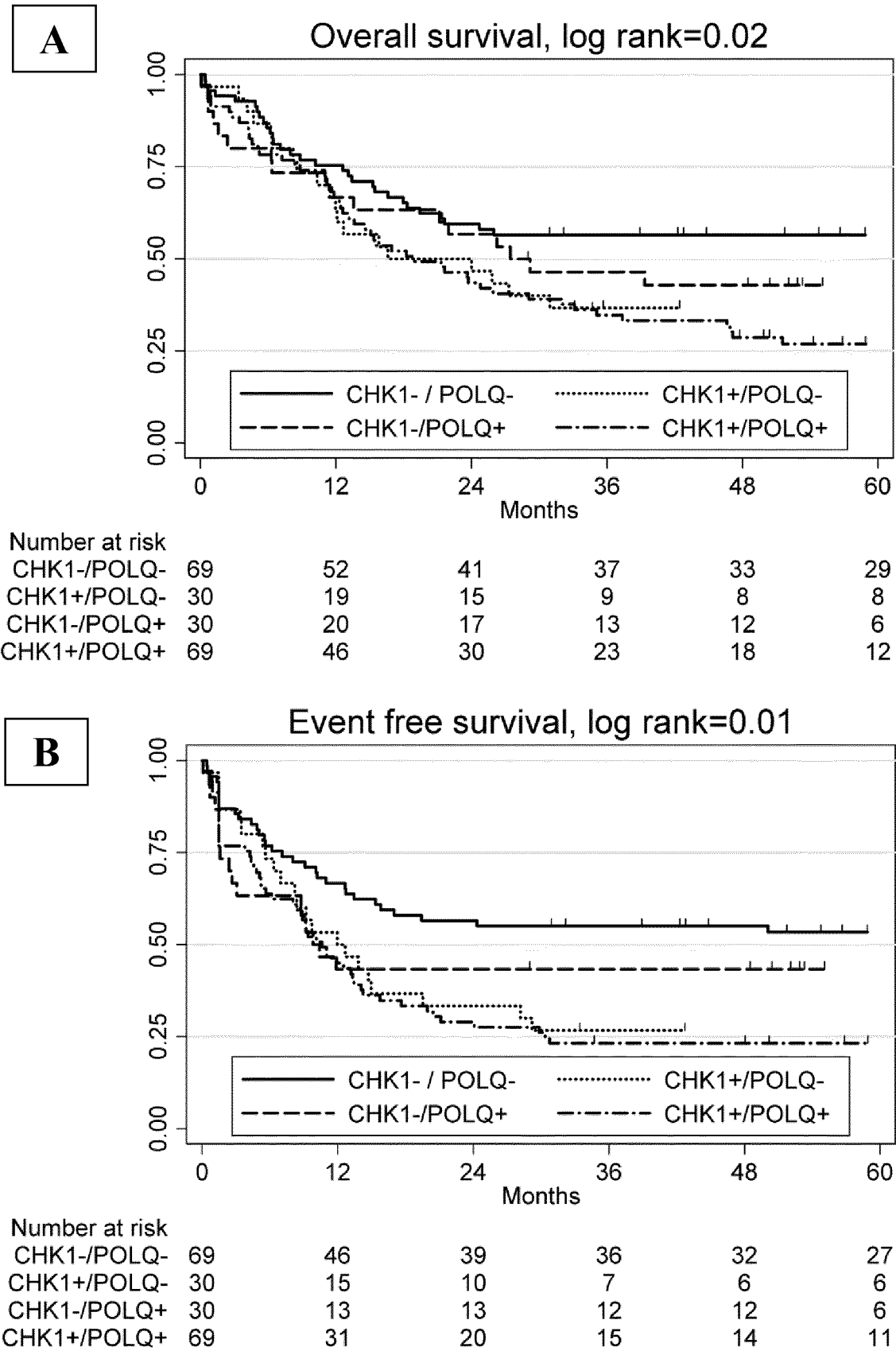


Figure 3A and B

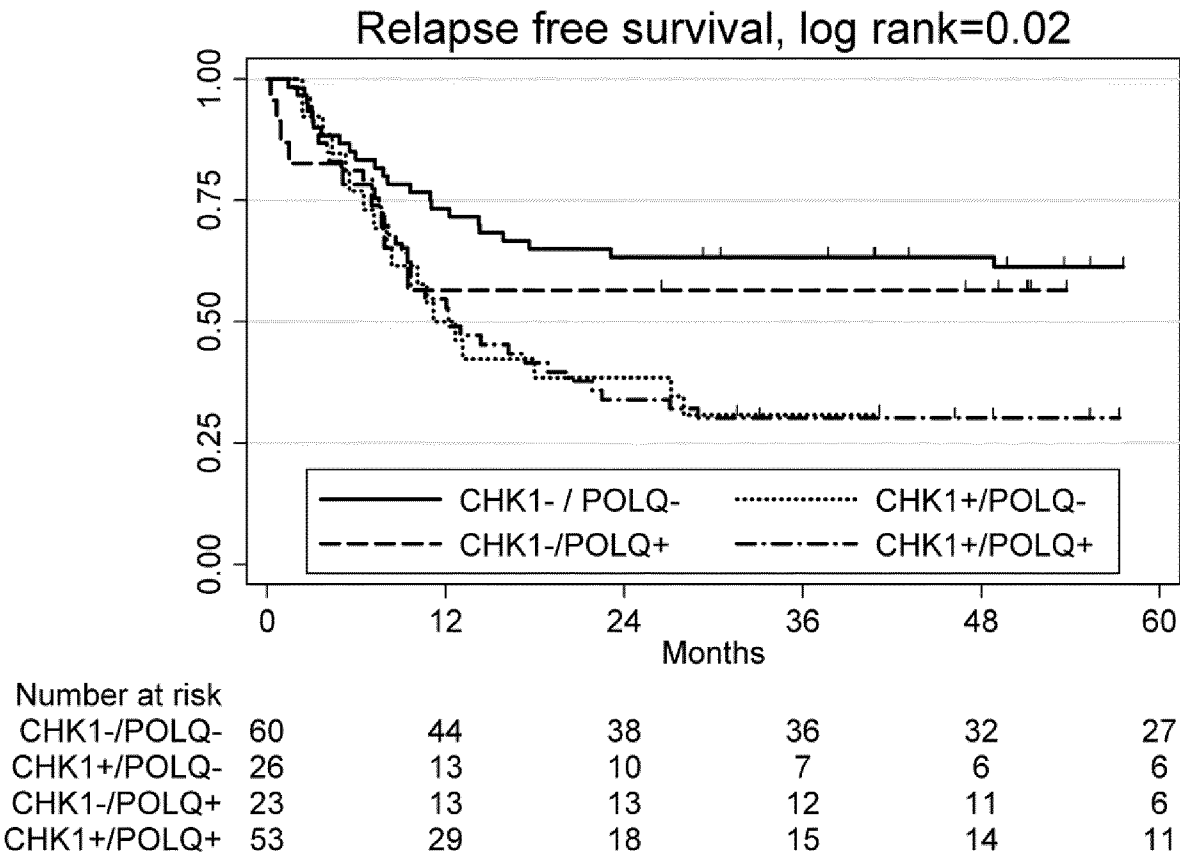


Figure 3 C

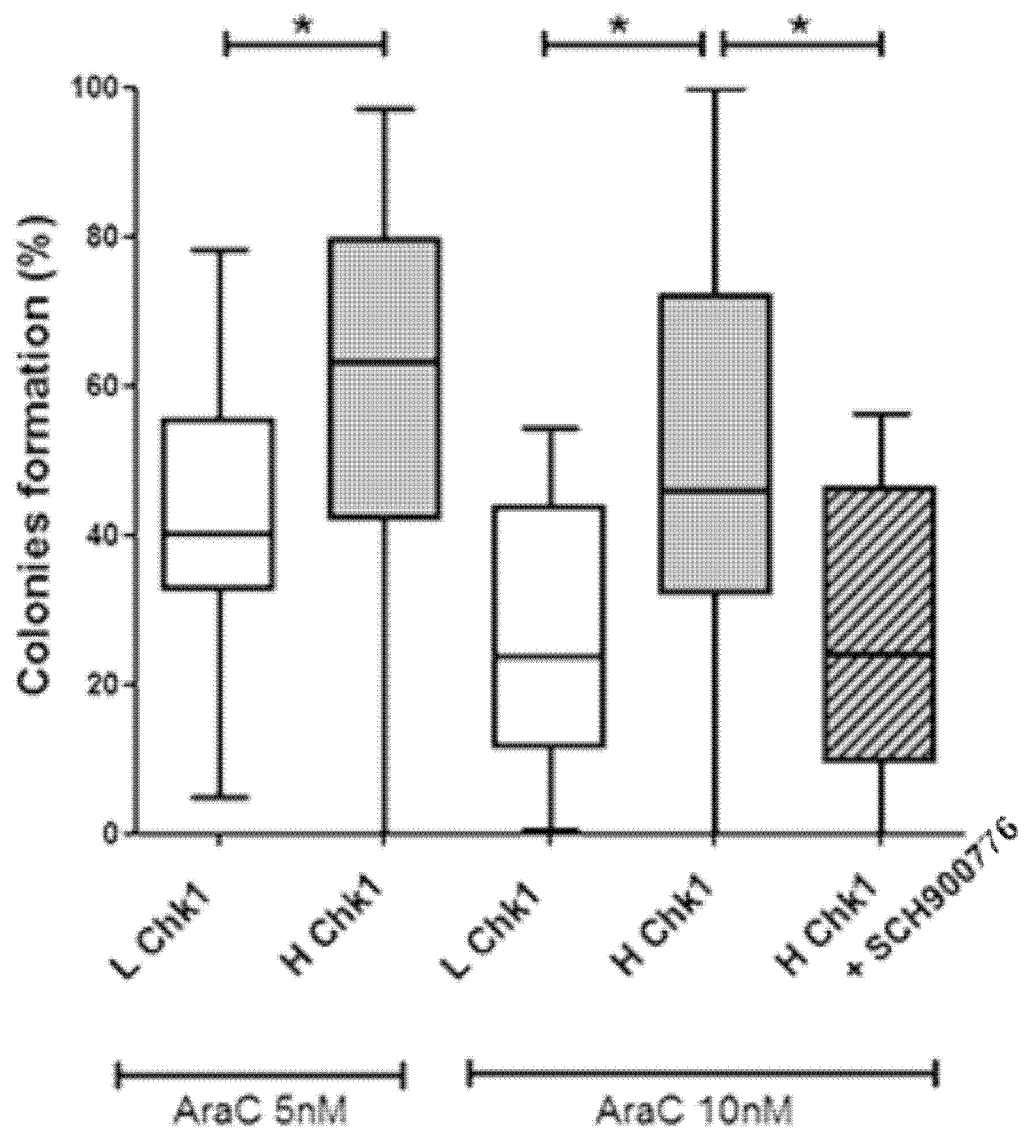


Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/053532

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 A61K38/20
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 A61K

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, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Merck Sharp & Dohme Corporation: "A Phase 1 Dose-escalation Study of a Cell Cycle Inhibitor With and Without Cytarabine in Patients With Acute Leukemias (Study P05247)(Terminated)", ClinicalTrials.gov, 26 September 2013 (2013-09-26), XP002727563, Retrieved from the Internet: URL:http://clinicaltrials.gov/show/NCT00907517 [retrieved on 2014-07-22]	1,2,6,7, 10
Y	abstract ----- -/--	3-5,8,9, 11,12



Further documents are listed in the continuation of Box C.



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

21 May 2015

Date of mailing of the international search report

01/06/2015

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Reuter, Uwe

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/053532

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XIE C ET AL.: "Panobinostat enhances cytarabine and daunorubicin sensitivities in AML cells through suppressing the expression of BRCA1, CHK1, and Rad51", PLOS ONE, vol. 8, no. 11, E79106, November 2013 (2013-11), pages 1-12, XP002727564,	1,2,6,10
Y	the whole document	3-5,8,9, 11,12
X	----- SCHENK E L ET AL.: "Effects of selective checkpoint kinase 1 inhibition on cytarabine cytotoxicity in acute myelogenous leukemia cell in vitro", CLINICAL CANCER RESEARCH, vol. 18, no. 19, October 2012 (2012-10), pages 5364-5373, XP002727565,	6,7,10
Y	the whole document	1-5,8,9, 11,12
X	----- KARP J E ET AL.: "Phase I and pharmacologic trial of cytosine arabinoside with the selective checkpoint 1 inhibitor Sch 900776 in refractory acute leukemias", CLINICAL CANCER RESEARCH, vol. 18, no. 24, 2012, pages 6723-6731, XP002727566,	6,7,10
Y	the whole document	1-5,8,9, 11,12
X	----- CAVELIER C ET AL.: "Constitutive activation of the DNA damage signaling pathway in acute myeloid leukemia with complex karyotype: potential importance for checkpoint targeting therapy", CANCER RESEARCH, vol. 69, no. 22, 15 November 2009 (2009-11-15), pages 8652-8661, XP002727567,	6,7,10
Y	the whole document	1-5,8,9, 11,12
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Y	cited in the application the whole document	1-5,8,9, 11,12
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/053532

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. BLASINA ET AL: "Breaching the DNA damage checkpoint via PF-00477736, a novel small-molecule inhibitor of checkpoint kinase 1", MOLECULAR CANCER THERAPEUTICS, vol. 7, no. 8, 1 August 2008 (2008-08-01), pages 2394-2404, XP055034300, ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-07-2391	6,7,10
Y	the whole document	1-5,8,9, 11,12
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Y	abstract; table 1 page 2125, column 1, last paragraph	1-5,8,9, 11,12
Y	----- BERTOLI S ET AL.: "Time from diagnosis to intensive chemotherapy initiation does not adversely impact the outcome of patients with acute myeloid leukemia", BLOOD, vol. 121, no. 14, 4 April 2013 (2013-04-04), pages 2618-2626, XP002727571, cited in the application the whole document	1-5,8,9, 11,12
A	----- F. LEMEE ET AL: "DNA polymerase up-regulation is associated with poor survival in breast cancer, perturbs DNA replication, and promotes genetic instability", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 107, no. 30, 27 July 2010 (2010-07-27), pages 13390-13395, XP055008221, ISSN: 0027-8424, DOI: 10.1073/pnas.0910759107 page 13394, right-hand column -----	1-12