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(54) Title: PROGNOSIS METHOD OF LEUKEMIA

(57) Abstract: The invention relates to a method for determining *in vitro* drug-resistance of cells of a leukemic sample, said method comprising, a step of evaluating, in cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins.



Description

Title of Invention: Prognosis method of leukemia

- [0001] The invention relates to a prognosis of leukemia, in particular a prognosis of resistance to drug that can be used to treat leukemic patients.
- [0002] Being able to predict cancer response to therapies is critical to increase response rates and limit their toxicity and, when necessary, change them.
- [0003] In this context, the inventors identified a modifomic signature based on protein post-translational modifications by Ubiquitin and the Ubiquitin-like SUMO polypeptides that may allow rapid discrimination of chemosensitive versus chemoresistant Acute Myeloid Leukemia (AML) at diagnosis.
- [0004] Most AML patients first receive an induction chemotherapy that has not significantly changed for the past 40 years (*Dombret H, Gardin C. Blood 2016; 127: 53–61*). It consists of two genotoxics, one anthracyclin (daunorubicin-DNR or idarubicin-IDA) and cytarabine (Ara-C). Unfortunately, a significant fraction of patients (20-30%) do not respond to this treatment and, among those achieving complete remission, relapse rates are high (5-years survival of 40% in patients below 60 and 20% in older ones - *Estey EH. Am J Hematol 2012; 87: 89–99*).
- [0005] To date, no prognosis tool implementable at diagnosis is available to predict AML response to chemotherapies. This is detrimental for at least two reasons: (i) unnecessary treatment of chemoresistant patients with anthracyclins that have life-threatening cardiotoxic effects and (ii) loss of time before redirecting patients to novel therapies (FLT3, IDH1/2, Bcl2 inhibitors...) and/or enrolling them in clinical trials for new molecules.
- [0006] Transcriptomic signatures are now used to better stratify patients and adapt treatments in several cancers. However, even though various transcriptomic signatures have been defined for AMLs, none of them is sufficiently reliable to be used in clinical practice. Interestingly, proteomic signatures, in particular those based on mass spectrometry, are emerging as promising alternatives. Even closer to the biological functions deregulated in cancer, « modifomic » analyses, which monitor the activities of enzymes involved in the post-translational modifications of proteins, open new perspectives in cancer prognosis/diagnosis.
- [0007] So there is a need to provide a prediction of cancer response to therapies, in order to ameliorate patient outcome, and to avoid the drawbacks known in the art.
- [0008] One aim of the invention is to provide a method that allows the practitioner to determine the best therapy to carry out faced to a patient.
- [0009] Another aim of the invention is to provide kits that could help the practitioner's choice when intending to provide the best therapy to the patients.

- [0010] For this purpose, the invention relates to a method for, or of, determining, preferably *in vitro*, drug-resistance of cells of a leukemic sample obtained from a patient afflicted by a leukemia, said method comprising:
- [0011] a. a step of evaluating, in cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins;
- [0012] wherein said at least 4 proteins correspond to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins correspond to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
- [0013] a. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said at the least 4 proteins of cells in said leukemic sample corresponding to a reference level, said reference level being the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins of leukemic cells that are sensitive to said drug in order to obtain a ratio of the level in the leukemic sample compared to the reference level of a sample of sensitive cells for each of said at least 4 proteins
- [0014] said reference level corresponding to the mean of the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins of several, possibly independent, leukemic cells that are sensitive to said drug; and
- [0015] a. a step of determining that
- [0016] – if the ratio for at least one of said 4 proteins is higher than 1.25 or lower than 0.8, then leukemic sample will be resistant to said drug, and
- if the ratio for each of said at least 4 proteins is comprised from 0.8 to 1.25, then leukemic sample will be sensitive to said drug.
- [0017] The invention is based on the unexpected observation made by the inventors that the evaluation of modifications by the enzymes belonging to the ubiquitin/Sumo pathways of a specific and determined group of 122 proteins, and in particular at least 4 of them, gives significant information regarding a resistance of a tumor to a determined drug.
- [0018] As exposed in the Example below, the inventors identified a group of 122 proteins that constitute group of proteins that are modified by ubiquitin/SUMO proteins and said modification being informative regarding the resistance to a drug. More particularly, the information which is important in the variation of the modification of said proteins by SUMO or ubiquitin.
- [0019] The group of 122 proteins according to the invention consists of the following proteins:

[0020]

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[Table 1]

Name	Accession .Number	Ultimate. ORF.ID. ..Catalog. Number	Gene. Symbol	SEQ ID
ataxin 3 (ATXN3)	BC033711.1	IOH21762	ATXN3	SEQ ID NO: 1
sequestosome 1 (SQSTM1)	BC001874.1	IOH4836	SQSTM1	SEQ ID NO: 2
UBA domain containing 1 (UBADC1)	BC004967.1	IOH4830	UBADC1	SEQ ID NO: 3
signal transducing adaptor molecule (SH3 domain and ITAM motif) 1 (STAM)	BC030586.2	IOH22241	STAM	SEQ ID NO: 4
Protein BEAN	XM_375359.1	IOH43383	BEAN	SEQ ID NO: 5
Optineurin	NM_001008211.1	IOH57143	OPTN	SEQ ID NO: 6
Nuclear protein localization protein 4 homolog	BC025930.1	IOH57231	NPLOC4	SEQ ID NO: 7
DCN1, defective in cullin ned- dylation 1, domain containing 1 (S. cerevisiae) (DCUN1D1)	BC009478.1	IOH12273	DCUN1D1	SEQ ID NO: 8
chromosome 9 open reading frame 71 (C9orf71)	NM_153237.1	IOH22672	C9orf71	SEQ ID NO: 9

polymerase (RNA) III (DNA directed) polypeptide K, 12.3 kDa (POLR3K)	NM_016310.2	IOH12571	POLR3K	SEQ ID NO: 10
ring finger protein 34 (RNF34), transcript variant 2	NM_025126.2	IOH6675	RNF34	SEQ ID NO: 11
zinc finger, MYM-type 5 (ZMYM5)	BC007048.1	IOH7230	ZMYM5	SEQ ID NO: 12
arrestin domain containing 1 (ARRDC1)	NM_152285.1	IOH21698	ARRDC1	SEQ ID NO: 13
hepatocyte growth factor- regulated tyrosine kinase substrate (HGS)	NM_004712.3	IOH4952	HGS	SEQ ID NO: 14
tripartite motif- containing 39 (TRIM39), transcript variant 2	NM_172016.1	IOH5913	TRIM39	SEQ ID NO: 15
DnaJ (Hsp40) homolog, subfamily B, member 2 (DNAJB2)	BC047056.1	IOH27319	DNAJB2	SEQ ID NO: 16
phospholipid scramblase 1 (PLSCR1)	NM_021105.1	IOH13995	PLSCR1	SEQ ID NO: 17
huntingtin in-	NM_005339.3	IOH13913	HIP2	SEQ ID NO: 18

teracting protein 2 (HIP2)				
NIMA (never in mitosis gene a)- related kinase 10 (NEK10)	BC045758.1	IOH26432	NEK10	SEQ ID NO: 19
ring finger protein 11 (RNF11)	NM_014372.1	IOH11235	RNF11	SEQ ID NO: 20
Isocitrate dehy- drogenase [NADP] cy- toplasmic	NM_005896.2	IOH11942	IDH1	SEQ ID NO: 21
Ubiquitin-conjug ating enzyme E2 D2	NM_003339.2	IOH58973	UBE2D2	SEQ ID NO: 22
FAS-associated factor 1	NM_007051.2	IOH5135	FAF1	SEQ ID NO: 23
ubiquitin-activati ng enzyme E1 (UBE1), transcript variant 1	NM_003334.2	IOH22897	UBE1	SEQ ID NO: 24
C-C motif chemokine 21	NM_002989.2	PHC1475	6CKINE	SEQ ID NO: 25
kinesin family member 3C (KIF3C)	NM_002254.5	IOH42589	KIF3C	SEQ ID NO: 26
HSPB (heat shock 27kDa) as- sociated protein 1 (HSPBAP1)	NM_024610.2	IOH13320	HSPBAP1	SEQ ID NO: 27
chromosome 5 open reading frame 32	BC023982.1	IOH14039	C5orf32	SEQ ID NO: 28

(C5orf32)				
SMAD family member 1 (SMAD1), transcript variant 1	NM_005900.1	IOH4970	SMAD1	SEQ ID NO: 29
lysozyme-like 1 (LYZL1)	NM_032517.3	IOH10985	LYZL1	SEQ ID NO: 30
Uncharacterized protein C4orf32	BC022534.1	IOH10832	C4orf32	SEQ ID NO: 31
SCO cytochrome oxidase deficient homolog 1 (yeast) (SCO1), nuclear gene encoding mitochondrial protein	NM_004589.1	IOH10823	SCO1	SEQ ID NO: 32
tetraspanin 9 (TSPAN9)	NM_031285.1	IOH22841	TSPAN9	SEQ ID NO: 33
OPN / SPP1 Protein (His Tag)	NP_001035147.1	10352-H08H-25	SPP1	SEQ ID NO: 34
Immunoglobulin iota chain	BC017922.1	IOH12726	VPREB1	SEQ ID NO: 35
Uncharacterized serine/threonine-protein kinase SgK494	NM_144610.1	IOH60533	FLJ25006	SEQ ID NO: 36
tripartite motif-containing 52 (TRIM52)	NM_032765.1	IOH7015	TRIM52	SEQ ID NO: 37
Thyroid hormone receptor beta	NM_000461.3	IOH29857	LBD	SEQ ID NO: 38
UBX domain containing 8	NM_014613.1	IOH40633	UBXD8	SEQ ID NO: 39

(UBXD8)				
protein phosphatase 2A, regulatory subunit B' (PR 53) (PPP2R4), transcript variant 4	NM_178002.1	IOH45891	PPP2R4	SEQ ID NO: 40
glycine-N-acyltransferase-like 2 (GLYATL2)	BC016789.1	IOH13602	GLYATL2	SEQ ID NO: 41
Transcriptional adapter 2-alpha	BC001172.1	IOH4467	TADA2L	SEQ ID NO: 42
phosphoserine aminotransferase 1 (PSAT1), transcript variant 1	NM_058179.1	IOH4946	PSAT1	SEQ ID NO: 43
hypothetical protein MGC52498 (MGC52498)	NM_182621.1	IOH28035	MGC52498	SEQ ID NO: 44
AKT interacting protein (AKTIP), transcript variant 2	NM_022476.1	IOH3844	AKTIP	SEQ ID NO: 45
ubiquitin-conjugating enzyme E2D1 (UBC4/5 homolog, yeast) (UBE2D1)	NM_003338.2	IOH7482	UBE2D1	SEQ ID NO: 46
Ubiquitin-like protein 7	NM_032907.3	IOH56973	UBL7	SEQ ID NO: 47
RIO kinase 3 (yeast) (RIOK3)	NM_003831.1	IOH20968	RIOK3	SEQ ID NO: 48

phospholipase A2, group IVD (cytosolic) (PLA2G4D)	NM_178034.1	IOH21503	PLA2G4D	SEQ ID NO: 49
glycoprotein, synaptic 2 (GPSN2)	NM_138501.3	IOH4169	GPSN2	SEQ ID NO: 50
capping protein (actin filament) muscle Z-line, beta (CAPZB)	BC008095.1	IOH3312	CAPZB	SEQ ID NO: 51
Ubiquitin-conjug ating enzyme E2 D3	BC066917.1	IOH61482	UBE2D3	SEQ ID NO: 52
OTU domain, ubiquitin aldehyde binding 2 (OTUB2)	BC000208.1	IOH4373	OTUB2	SEQ ID NO: 53
polymerase (RNA) II (DNA directed) polypeptide H (POLR2H)	NM_006232.2	IOH4626	POLR2H	SEQ ID NO: 54
ubiquilin 1 (UBQLN1), transcript variant 2	NM_053067.1	IOH40632	UBQLN1	SEQ ID NO: 55
GRIP1-associate d protein 1	NM_020137.3	IOH52884	GRIPAP1	SEQ ID NO: 56
ribosomal protein S17 (RPS17)	NM_001021.2	IOH27847	RPS17	SEQ ID NO: 57
ubiquitin-conjuga ting enzyme E2 variant 1 (UBE2V1)	BC000468.1	IOH3618	UBE2V1	SEQ ID NO: 58

adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL1)	NM_012096.1	IOH11629	APPL1	SEQ ID NO: 59
MOB1, Mps One Binder kinase activator-like 2C (yeast) (MOBKL2C), transcript variant 2	NM_201403.1	IOH42184	MOBKL2C	SEQ ID NO: 60
UBX domain containing 1 (UBXD1)	NM_025241.1	IOH41143	UBXD1	SEQ ID NO: 61
ubiquitin-conjugating enzyme E2E2 (UBC4/5 homolog, yeast) (UBE2E2)	NM_152653.1	IOH13176	UBE2E2	SEQ ID NO: 62
ankyrin repeat domain 37 (ANKRD37)	NM_181726.1	IOH43299	ANKRD37	SEQ ID NO: 63
aldehyde dehydrogenase 2 family (mitochondrial) (ALDH2), nuclear gene encoding mitochondrial protein	NM_000690.1	IOH4924	ALDH2	SEQ ID NO: 64
lysozyme-like 2 (LYZL2)	BC066294.1	IOH40058	LYZL2	SEQ ID NO: 65
Uncharacterized	BC020885.1	IOH14357	C12orf65	SEQ ID NO: 66

protein C12orf65				
tetraspanin 1 (TSPAN1)	NM_005727.2	IOH6907	TSPAN1	SEQ ID NO: 67
spleen tyrosine kinase (SYK)	NM_003177.3	PV3857	SYK	SEQ ID NO: 68
WD repeat domain 88 (WDR88)	BC031227.1	IOH21600	WDR88	SEQ ID NO: 69
chromosome 2 open reading frame 34 (C2orf34)	BC053733.1	IOH28787	C2orf34	SEQ ID NO: 70
EP300-interactin g inhibitor of dif- ferentiation 3	BC027612.2	IOH11844	EID3	SEQ ID NO: 71
cysteine and histidine-rich domain (CHORD)-contai ning 1 (CHORDC1)	BC017789.1	IOH13662	CHORDC1	SEQ ID NO: 72
acyl-CoA synthetase medium-chain family member 3 (ACSM3), transcript variant 1	NM_005622.3	IOH42133	ACSM3	SEQ ID NO: 73
ubiquitin-conjuga ting enzyme E2W (putative) (UBE2W)	BC010900.1	IOH12898	UBE2W	SEQ ID NO: 74
PREDICTED: Homo sapiens hypothetical protein	XM_379183.1	IOH43202	LOC15202 4	SEQ ID NO: 75

LOC152024 (LOC152024)				
Gap junction beta-2 protein	BC071703.1	IOH61781	GJB2	SEQ ID NO: 76
Exocyst complex component 5 (EXOC5), mRNA	NM_006544.3	IOH55643	EXOC5	SEQ ID NO: 77
complement component 1, q subcomponent, C chain (C1QC)	BC009016.1	IOH3326	C1QC	SEQ ID NO: 78
ankyrin repeat and sterile alpha motif domain containing 4B (ANKS4B)	NM_145865.1	IOH35802	ANKS4B	SEQ ID NO: 79
RAD23 homolog A (<i>S. cerevisiae</i>) (RAD23A)	NM_005053.2	IOH22983	RAD23A	SEQ ID NO: 80
Leukocyte im- munoglobulin-lik e receptor, subfamily A (with TM domain), member 4 (LILRA4), mRNA	NM_012276.3	IOH43377	LILRA4	SEQ ID NO: 81
Potassium voltage-gated channel, shaker- related subfamily, member 4 (KCNA4), mRNA	NM_002233.1	IOH29649	KCNA4	SEQ ID NO: 82

rhomboid domain containing 1 (RHBDD1)	BC027900.1	IOH27306	RHBDD1	SEQ ID NO: 83
RAD18 homolog (<i>S. cerevisiae</i>) (RAD18)	NM_020165.2	IOH3060	RAD18	SEQ ID NO: 84
inversin (INVS)	BC006370.2	IOH6467	INVS	SEQ ID NO: 85
chromosome 6 open reading frame 106 (C6orf106), transcript variant 1	NM_024294.1	IOH5168	C6orf106	SEQ ID NO: 86
Cbl-interacting protein Sts-1 (STS-1)	NM_032873.3	IOH6812	STS-1	SEQ ID NO: 87
Rho GTPase activating protein 4 (ARHGAP4)	BC052303.1	IOH28113	ARHGAP4	SEQ ID NO: 88
Histone 1, H1c (HIST1H1C), mRNA	NM_005319.3	IOH5275	HIST1H1C	SEQ ID NO: 89
chromosome 13 open reading frame 24 (C13orf24)	BC051911.1	IOH27047	C13orf24	SEQ ID NO: 90
myosin IXA (MYO9A)	BC060886.1	IOH29219	MYO9A	SEQ ID NO: 91
Cell division cycle 2-like protein kinase 5	NM_003718.2	PMX_10184	CDC2L5	SEQ ID NO: 92
Putative phosphatidylinositol 4-kinase alpha-like protein P2	BC020225.1	IOH13323	LOC220686	SEQ ID NO: 93

Inhibitor of nuclear factor kappa-B kinase subunit epsilon	NM_014002.1	--	IKBKE	SEQ ID NO: 94
Myosin light chain 2, lymphocyte-specific	BC002778.1	IOH5313	MYLC2PL	SEQ ID NO: 95
splicing factor 3b, subunit 2, 145kDa (SF3B2)	BC007610.1	IOH6911	SF3B2	SEQ ID NO: 96
parathymosin (PTMS)	BC017025.1	IOH9960	PTMS	SEQ ID NO: 97
Follistatin (FST), transcript variant FST317, mRNA	NM_006350.2	IOH59070	FST	SEQ ID NO: 98
a serine/threonine kinase	NM_004958.2	PV4753	FRAP1 (mTOR)	SEQ ID NO: 99
similar to 60S ribosomal protein L21 (LOC402176)	NM_001011538.1	IOH39826	LOC402176	SEQ ID NO: 100
SDF1B Re-combinant Human Protein	NP_000600.1	PHC1361	SDF1B	SEQ ID NO: 101
SUMO1 activating enzyme subunit 2 (SAE2)	NM_005499.2	IOH5085	SAE2	SEQ ID NO: 102
Transmembrane protein 207	NM_207316.1	IOH45939	UNQ846	SEQ ID NO: 103
Chromosome 18 open reading frame 1 (C18orf1),	NM_001003675.1	IOH53461	LDLRAD4	SEQ ID NO: 104

transcript variant c2, mRNA				
ubiquitin specific peptidase 5 (isopeptidase T) (USP5)	BC005139.2	IOH4709	USP5	SEQ ID NO: 105
Prolyl 4-hydroxylase subunit alpha-2	NM_001017973.1	IOH26049	P4HA2	SEQ ID NO: 106
Triosephosphate isomerase 1, mRNA (cDNA clone MGC:88108 IMAGE:6725406 , complete cds	BC070129.1	IOH63021	TPI1	SEQ ID NO: 107
Rho-related BTB domain containing 1 (RHOBTB1), transcript variant 1	NM_014836.3	IOH27118	RHOBTB1	SEQ ID NO: 108
KIAA0247 (KIAA0247)	NM_014734.2	IOH39813	KIAA0247	SEQ ID NO: 109
transmembrane protein 174 (TMEM174)	NM_153217.1	IOH13241	TMEM174	SEQ ID NO: 110
zinc finger, AN1-type domain 5 (ZFAND5), transcript variant c	NM_006007.1	IOH14289	ZFAND5	SEQ ID NO: 111
ring finger protein 128 (RNF128),	NM_194463.1	IOH39866	RNF128	SEQ ID NO: 112

transcript variant 1				
TOM1-like protein 2	NM_001033551.1	IOH26533	TOM1L2	SEQ ID NO: 113
Spermine oxidase	NM_019025.2	IOH41563	SMOX	SEQ ID NO: 114
ubiquitin-conjugating enzyme E2D3 (UBC4/5 homolog, yeast) (UBE2D3), transcript variant 8	NM_181892.1	IOH43701	UBE2D3	SEQ ID NO: 115
Ubiquilin-4	BC006410.1	IOH6249	UBQLN4	SEQ ID NO: 116
ring finger protein 130 (RNF130)	BC017100.1	IOH12953	RNF130	SEQ ID NO: 117
proteasome (prosome, macropain) 26S subunit, non-ATPase, 4 (PSMD4)	NM_002810.1	IOH3462	PSMD4	SEQ ID NO: 118
F-box/WD repeat-containing protein 11	BC026213.1	IOH11042	FBXW11	SEQ ID NO: 119
ring finger protein 34 (RNF34), transcript variant 1	NM_194271.1	IOH41584	RNF34	SEQ ID NO: 120
zinc finger, CCHC domain containing 12 (ZCCHC12)	BC036572.2	IOH28725	ZCCHC12	SEQ ID NO: 121

GTP-binding nuclear protein Ran	NM_006325.2	IOH10063	RAN	SEQ ID NO: 122
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- [0021] In the method according to the invention, the first step intends to evaluate the modification of at least the proteins consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, by ubiquitin or SUMO, or ubiquitin and SUMO proteins, in order to obtain a value of modification by one or two of said Ubiquitin and SUMO proteins.
- [0022] In the method mentioned above, it can be evaluated the variation of the modifications by SUMO and/or ubiquitin of 4 proteins consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, or any group of at least 4 proteins, i.e. 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121 or 122 protein, provided that said group comprises at least the proteins consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4.
- [0023] Such modifications by ubiquitin or SUMO can be easily evaluated by immunoblotting assays using specific antibodies directed respectively against Ubiquitin or SUMO proteins. these proteins are the markers of the activity of the enzymes involved in the Ubiquitin/Sumo pathways, which are present in the leukemic samples.
- [0024] The at the least 4 proteins chosen among the group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins, are contacted with the leukemic samples such that the deregulation of the Ubiquitin/Sumo pathways in the leukemic sample is detected by variations in the levels of modification by ubiquitination or sumoylation of said at least 23 proteins.
- [0025] For instance, the proteins which are modified by ubiquitin and/or SUMO proteins will therefore interact with antibodies directed against ubiquitin and/or SUMO proteins to form a molecular complex. The complexes can be detected by secondary antibodies that recognize (and interact with) constant part FC chain of said antibodies directed against ubiquitin and/or SUMO proteins.
- [0026] Said complex can be identified when the secondary antibodies are labeled with reporter molecule, such as fluorescent protein, peroxidase, fluorescent dyes etc...
- [0027] The skilled person knows how to quantify the complexes protein/anti ubiquitin-SUMO/ anti-antibodies, in particular by using flow cytometers.

- [0028] An advantageous method is disclosed (XMap bead-based assay) in the Example below. $2 \cdot 10^6$ cells are resuspended and incubated at 4 °C for 30 min in 25 μ L of a hypotonic buffer (20 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT and 1 mg/L of aprotinine, leupeptin and pepstatin). Cell lysis is achieved through 4 freezing/thawing cycles using liquid nitrogen and DNA was sheared owing to 10 passages through a 20-1/2G needle. Extracts are finally centrifuged twice (16000 g) at 4°C for 20 min and supernatants were aliquoted, flash-frozen and kept at -80 °C until use. SUMO-1-, SUMO-2- and Ubiquitin vinyl sulfones (0.5 μ M each) are added to 10 μ L of cellular extracts and incubated at 4 °C for 15 min. XMap beads (Luminex) coupled to the proteins mentioned above (one protein per bead) are then added to the extract in 10 μ L of a reaction buffer containing 20 mM HEPES pH 7.3, 110 mM KOAc, 2 mM $Mg(OAc)_2$, 0.05 % Tween-20, 0.5 mM EGTA, 0.2 mg/mL ovalbumine, 1 mM DTT, 1 mg/L aprotinin, leupeptin and pepstatin, 1 mM ATP, 30 μ M Flag-ubiquitin, 15 μ M SUMO-1 and 15 μ M SUMO-2. Reaction are performed at 30 °C for 45 min. Beads are washed twice for 5 min with PBS containing 0.05 % Tween-20 and 0.5 % SDS and 3 times for 5 min with PBS containing 0.05 % Tween-20. They are then incubated with 1 μ g/mL of anti-SUMO-1 (21C7) and anti-Flag antibodies for 1 hr under agitation at room temperature. After washing in PBS containing 0.05% Tween-20 for 5 min, they are incubated for 30 min at room temperature with anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 405 antibodies in 100 μ L of PBS containing 0.05 % Tween-20. Beads are again washed for 5 min with PBS containing 0.05 % Tween-20. They are then resuspended in 200 μ L PBS and flow-cytometry-analysed.
- [0029] The detection of the above-mentioned complexes allows to establish a value of modification which corresponds to the Ubiquitin/SUMO modification status of said at least 4 proteins for each leukemic samples of unknown response to said drug.
- [0030] The value is calculated for each of said proteins and can be noted as VC_i , wherein i corresponds to the SEQ ID of said at least 4 proteins.
- [0031] Thus, when only the modification status of said 4 proteins is evaluated, 4 values are established:
- [0032] – a value for SEQ ID NO: 1 – VC_1 ;
– a value for SEQ ID NO: 2 – VC_2 ;
– a value for SEQ ID NO: 3 – VC_3 ; and
– a value for SEQ ID NO: 4 – VC_4 .
- [0033] In order to normalize the value calculated in the step a), it is also calculated the value of the modification by ubiquitin and SUMO of said at least 4 proteins in leukemic cells that are known to be sensitive to said drug. These cells correspond to the reference cells.
- [0034] Drug sensitivity of said control cells can be determined experimentally, or is known

either by nature (the cells were selected to be sensitive to said drug), or can be obtained from patient afflicted by a leukemia that was successfully treated with said drug.

[0035] It is advantageous that the value VR_i be the mean of VR_i obtained in many cells, in order to intend to avoid any variability. It is also advantageous to use the same control cells to calculate the VR_i for each of said at least 4 proteins, in order to have a homogenous reference.

[0036] Said value can be established for each protein as VR_i , wherein i corresponds to the SEQ ID of said at least 4 proteins.

[0037] Thus, when only the modification status of said 4 proteins is evaluated, 4 references values are established:

- [0038] – a value for SEQ ID NO: 1 – VR_1 ;
– a value for SEQ ID NO: 2 – VR_2 ;
– a value for SEQ ID NO: 3 – VR_3 ; and
– a value for SEQ ID NO: 4 – VR_4 .

[0039] It is therefore established a ratio R_i for each protein such that $R_i = VC_i/VR_i$, wherein i corresponds to the SEQ ID of said at least 4 proteins.

[0040] When the ratio are established, it is possible to determine that

- [0041] – if all the ratio R_i , calculated for each of said at least 4 proteins, are comprised between 0.8 and 1.25, thus the leukemic cells that are studied are considered to be sensitive to said drug; and
– if at least one ration R_i is higher than 1.25, or lower to 0.8, then the leukemic cells that are studied are considered to be resistant to said drug.

[0042] When considering only the 4 proteins of SEQ ID NO: 1 to SEQ ID NO: 4, the ratio R_1 , R_2 , R_3 and R_4 are calculated. If R_1 , R_2 , R_3 and R_4 are between 0.8 and 1.25, then the leukemic cells are considered to be sensitive to said drug.

[0043] On the contrary, when at least R_1 , or R_2 , or R_3 or R_4 is higher than 1.25, or lower to 0.8, then the leukemic cells are considered to be resistant to said drug.

[0044] As mentioned above, the ratio can represent an increase of the ubiquitin/SUMO modification in the leukemic cells resistant to said drug compared to the control leukemic cells that are sensitive to said drug. In such a case, an increase of the modification by ubiquitin or SUMO can represent the hallmark of resistance. The ratio can also represent a decrease of the ubiquitin/SUMO modification in the leukemic cells resistant to said drug compared to the control leukemic cells that are sensitive to said drug. In such a case, a decrease of the modification by ubiquitin or SUMO can represent the hallmark of resistance.

[0045] Advantageously, the invention relates to the method as defined above, wherein

[0046] - the protein consisting essentially or consisting of the sequence as set forth in SEQ ID NO: 1 (ATXN3) is modified by Sumoylation,

- [0047] - the protein consisting essentially or consisting of the sequence as set forth in SEQ ID NO: 2 (SQSTM1) is modified by Ubiquitination, and
- [0048] - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 3 (UBADC1) and SEQ ID NO: 4 (STAM) are modified by Ubiquitination and Sumoylation.
- [0049] The inventors identified that the protein as set forth in SEQ ID NO: 1 is only modified by SUMO protein. Therefore, the modification that can be evaluated for this protein corresponds only to an increase or a decrease of the amount of SUMO protein.
- [0050] The inventors also identified that the protein as set forth in SEQ ID NO: 2 is only modified by ubiquitin protein. Therefore, the modification that can be evaluated for this protein corresponds only to an increase or a decrease of the amount of ubiquitin protein.
- [0051] The inventors also identified that both proteins as set forth in SEQ ID NO: 3 and 4 are modified by both ubiquitin and SUMO proteins.
- [0052] Advantageously, the invention relates to the method as previously defined, wherein if the modification of the protein as set forth in SEQ ID NO: 2 or 3 or 4 is an ubiquitination, and if the ratio is higher than 1.25, then leukemic sample will be resistant to said drug.
- [0053] As mentioned above, the proteins as depicted in SEQ ID NO: 2, 3 and 4, are modified by ubiquitin. Therefore, the evaluation of the variation of ubiquitination of at least one of these proteins gives information regarding the resistance to said drug.
- [0054] More advantageously, the invention relates to the method as defined above, wherein if the modification of the protein as set forth in SEQ ID NO: 1 or 3 or 4 is a sumoylation, and if the ratio is higher than 1.25, then leukemic sample will be resistant to said drug.
- [0055] As mentioned above, the proteins as depicted in SEQ ID NO: 1, 3 and 4, are modified by SUMO. Therefore, the evaluation of the variation of SUMO of at least one of these proteins gives information regarding the resistance to said drug.
- [0056] More advantageously, the invention relates to the method as defined above, comprising a step a) of evaluating, in said leukemic sample, the level of modification by ubiquitination and/or sumoylation of each protein of said group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins.
- [0057] Advantageously, the inventors identified that if a group of 23 proteins is studied, i.e. if the variation of the amount of ubiquitin and/or SUMO of at least said 23 proteins represented by the proteins as set forth in SEQ ID NO: 1 to 23, the evaluation of the drug resistance or sensitivity is higher.
- [0058] Therefore, an advantageous embodiment of the invention relates to a method for, or of, determining, preferably *in vitro*, drug-resistance of cells of a leukemic sample

obtained from a patient afflicted by a leukemia, said method comprising:

- [0059] a. a step of evaluating, from cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 23 proteins, said group of 23 proteins belonging to a set of 122 proteins;
- [0060] wherein said at least 23 proteins correspond to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
- [0061] a. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said at the least 23 proteins of cells in said leukemic sample corresponding to a reference level, said reference level being the level of modification by ubiquitination and/or sumoylation of each at the least 23 proteins of leukemic cells that are sensitive to said drug in order to obtain a ratio of the level in the leukemic sample compared to the reference level of a sample of sensitive cells for each of said at least 23 proteins
- [0062] said reference level corresponding to the mean of the level of modification by ubiquitination and/or sumoylation of each at the least 23 proteins of several, possibly independent, leukemic cells that are sensitive to said drug; and
- [0063] a. a step of determining that
- [0064] – if the ratio for at least one of said 23 proteins is higher than 1.25 or lower than 0.8, then leukemic sample will be resistant to said drug, and
- if the ratio for each of said at least 23 proteins is comprised from 0.8 to 1.25, then leukemic sample will be sensitive to said drug.
- [0065] Advantageously, the invention relates to the method as defined above, wherein
- [0066] - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 20 are modified by Sumoylation,
- [0067] - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 17 to 19, SEQ ID NO: 21 and SEQ ID NO: 23 are modified by Ubiquitination, and
- [0068] - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 3 and 4, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 16 and SEQ ID NO: 22 are modified by Ubiquitination and Sumoylation.
- [0069] More advantageously, the invention relates to the method as defined above, comprising a step a) of evaluating, in said leukemic sample, the level of modification by ubiquitination and/or sumoylation of each protein of said set of 122 proteins.
- [0070] More advantageously, the inventors identified that the evaluation of ubiquitination/SUMOylation of the 122 proteins as depicted in SEQ ID NO: 1 to 122 gives the best

results in determining said drug resistance.

[0071] Thus, the invention relates advantageously to a method for, or of, determining, preferably *in vitro*, drug-resistance of cells of a leukemic sample obtained from a patient afflicted by a leukemia, said method comprising:

- [0072] a. a step of evaluating, from cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein a set of 122 proteins, wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
- b. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said 122 proteins of cells in said leukemic sample corresponding to a reference level, said reference level being the level of modification by ubiquitination and/or sumoylation of each 122 proteins of leukemic cells that are sensitive to said drug in order to obtain a ratio of the level in the leukemic sample compared to the reference level of a sample of sensitive cells for each of said 122 proteins

[0073] said reference level corresponding to the mean of the level of modification by ubiquitination and/or sumoylation of each 122 proteins of several, possibly independent, leukemic cells that are sensitive to said drug; and

[0074] a. a step of determining that

- [0075] – if the ratio for at least one of said 122 proteins is higher than 1.25 or lower than 0.8, then leukemic sample will be resistant to said drug, and
- if the ratio for each of said 122 proteins is comprised from 0.8 to 1.25, then leukemic sample will be sensitive to said drug.

[0076] More advantageously, the invention relates to the method as defined above, wherein said drug is chosen from anthracycline and/or cytarabine.

[0077] The anthracyclines are one of the most effective anticancer treatments ever developed and are effective against more types of cancer than any other class of chemotherapeutic agents. These drugs act mainly by intercalating with DNA and interfering with DNA metabolism and RNA production.

[0078] Cytarabine, also known as cytosine arabinoside (ara-C), is a chemotherapy medication used to treat main of the blood cancer, and acts by blocking the function of DNA polymerase.

[0079] More advantageously, the invention relates to the method as defined above, wherein said leukemia is an acute myeloid leukemia.

[0080] The invention relates to a method for, or of, the prognosis, preferably *in vitro*, of a leukemia in a sample of a patient comprising the following steps:

- [0081] a. a step of evaluating, from cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 4

proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins;

[0082] wherein said at least 4 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,

[0083] a. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said at the least 4 proteins in cells of said leukemic sample to the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins, in leukemic samples that are sensitive to said drugs, in order to obtain a ratio of the level in the leukemic sample compared to a panel of drug-sensitive-leukemic samples for each of at least 4 proteins; and
b. a step of determining that if the ratio of each of said at least 4 proteins is comprised from 0.8 to 1.25, then the patient could be treated with anthracyclin and/or cytarabine.

[0084] Advantageously, the invention relates to the method defined above, wherein in step c) if the ratio is lower than 0.8 or higher to 1.25 for at least one of said at least four proteins, then the patient should be treated with FLT3, IDH1/2, Bcl2 proteins inhibitors or enrolled in a clinical trial for new molecules.

[0085] Advantageously, the leukemia is an acute myeloid leukemia.

[0086] The invention also relates to a kit comprising:

[0087] – at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins, wherein said at least 4 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
– at least one antibody directed against Ubiquitin and at least one antibody directed against SUMO, and
– means for detecting the ubiquitination and/or sumoylation of said at least 4 proteins.

[0088] Advantageously, the invention relates to the kit as defined above, further comprising leukemic cells, or extracts of leukemic cells that are sensitive to a determined drug.

[0089] Advantageously, the invention relates to the kit as defined above, wherein said determined drug is anthracyclin and/or cytarabine.

- [0090] In other words, the invention relates advantageously to a kit comprising:
- [0091] – at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins, wherein said at least 4 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
- at least one antibody directed against Ubiquitin and at least one antibody directed against SUMO, and
- means for detecting the ubiquitination and/or sumoylation of said at least 4 proteins,
- and possibly further comprising leukemic cells, or extracts of leukemic cells that are sensitive a drug, possibly resistant to anthracyclin and/or cytarabine

Brief description of the drawings

- [0092] [Fig.1] represents the measurement of IC_{50} of ARA-C (cytarabine) and DNR (daunorubicin). HL-60 and U937 parental, resistant to Ara-C or DNR were cultured with increasing concentration of Ara-C or DNR and their IC_{50} was measured at 24 h.
- [0093] [Fig.2] Represents the procedure for the identification of UbL signature. Extracts from HL-60 or U937 (parental, resistant to DNR, resistant to Ara-C) were supplemented with UbL-vinyl-sulfone and recombinant UbL and incubated on the Protoarrays. After extensive washes, the arrays were incubated with primary mouse anti-SUMO-1 and rabbit anti-Flag (tag present on the recombinant Ubiquitin added to the reaction) antibodies followed by fluorescently coupled secondary antibodies. The arrays were then analysed using PAA package and statistical analysis performed to identify a UbL signature of chemoresistance. Three independent experiments were performed for each cell line.
- [0094] [Fig.3] Identification of the ubiquitylated and SUMOylated proteins. The normalized fluorescence data obtained for both modifiers on all arrays were compared to the averaged signal of the control arrays (NEM) to identify proteins, which are robustly modified. The proteins showing a significant difference between the two groups using both Welch and Wilcoxon-Mann-Whitney and having mean fluorescence intensities values higher than 800 on the Protoarrays were selected for further analysis.
- [0095] [Fig.4] Identification of UbL biomarkers of AML chemoresistance. The level of modification of the proteins modified by Ubiquitin (left panel) or SUMO-1 (central panel) selected in C was compared between the parental HL-60 and U937 and their resistant counterparts. Right panel, Venn diagram with proteins showing a significant

p-value in both Wilcoxon signed-rank test and one sample *t*-test and a ratio between resistant and parental cells higher than 1.25 or lower than 0.8.

- [0096] [Fig.5] Identification of UbL biomarkers specific for HL-60 and U937 resistance to Ara-C or DNR. The statistical analysis between resistant and parental cell lines was performed separately for U937 and HL-60 and for each drug resistance. The number of proteins showing a significant p-value in one sample *t*-test and a ratio between resistant and parental cells higher than 1.5 or lower than 0.66 are shown.
- [0097] [Fig.6] Ontology analysis of the UbL signature. An ontology analysis of the 122 proteins of the UbL signature was performed using the Panther software.
- [0098] [Fig.7] Principle of the assay to measure the UbL modification of the signature proteins. Recombinant proteins produced in bacteria are coupled to XMap beads, one protein per colored bead. The beads are multiplexed and incubated with cell extracts supplemented with UbL-vinyl-sulfone and recombinant UbL. After washing, the beads are incubated with antibodies directed against SUMO-1 and Flag (tag present on the recombinant Ubiquitin added to the reaction) and fluorescent secondary antibodies. The beads are then analysed by cytometry.
- [0099] [Fig.8] Analysis of UbL biomarkers modification. Extracts from HL-60 parental, resistant to Ara-C or DNR were used as described in A with beads coupled to ARDC1 or HIP2. Their ubiquitylation was monitored by flow cytometry. Representative cytometry profiles are shown (n=9 for ARDC1, n=3 for HIP2)
- [0100] [Fig.9] Validation of UbL biomarkers. The level of ubiquitylation of 14 of the signature proteins was compared between extracts from HL-60 parental or resistant to Ara-C or DNR. Only the 6 proteins showing significant differences between the parental and the resistant cells are shown (n=3 to 9 depending on the proteins).
- [0101] [Fig.10] Analysis of UbL biomarkers in patient samples. Extracts from bone marrow aspirates from 17 patients at diagnosis were used with multiplexed XMap beads coupled to different proteins of the UbL signature. Twelve patients responded to the induction chemotherapy (blue, less than 10% of blasts in bone marrow 30 days after the beginning of the chemotherapy) and 5 didn't respond (red, more than 10% of blasts in bone marrow 30 days after the beginning of the chemotherapy). Only the proteins showing a significant level of ubiquitylation are shown. The average intensity of the ubiquitylation is presented as a line for both responder and non-responder patients.
- [0102] [Fig.11] Figure 11 represents a graph showing the probability of acute myeloid leukemias sensitivity/resistance using a score based on the use of a genetic algorithm and a linear discriminant analysis. This score was used to predict the probability of resistance for the U937 and HL60 cell lines. Cells were considered sensitive to chemotherapy if the probability to belong to the group of resistant cells was below 50% and resistant if over 50%.

- [0103] [Fig.12] Figure 12 represents a graph showing the probability of acute myeloid leukemias sensitivity/resistance using the predictive score to predict the probability of resistance for 4 patient samples (2 responders and 2 non-responders). Cells were considered sensitive to chemotherapy if the probability to belong to the group of resistant cells was below 50% and resistant if over 50%.
- [0104] [Fig.13] Figure 13 represents graphs which are representative flow cytometry profiles for ubiquitylation.
- [0105] [Fig.14] Figure 14 represents the quantification of the modifications. For quantification, background (cell extracts supplemented with NEM to inhibit UbL conjugation activities) was subtracted and ratio between resistant and parental cell lines was shown (n = 6 for STAM, n = 3 for SQSTM1, UBADC1 and HIP2). Mean $\pm$ SEM. Paired t test, *P < 0.05, **P < 0.01, ***P < 0.001.
- [0106] [Fig.15] Extracts from bone marrow aspirates from 37 patients at diagnosis were used in a multiplexed flow cytometry analysis using xMap beads coupled to STAM, UBADC1, and SQSTM1. 29 patients responded to induction chemotherapy (<10% of blasts in bone marrow 30 d after the beginning of chemotherapy) and 10 did not (>10% of blasts in bone marrow 30 d after the beginning of chemotherapy). Mean $\pm$ SEM. Unpaired t test with Welch's correction. For the refractory patients, those showing high ubiquitylation for at least one of the biomarkers are grey-coded. MFI, median fluorescence intensity; ns, not significant.

Example

- [0107] Ubiquitin family proteins (collectively hereafter called UbLs) are peptidic post-translational modifiers. The best-characterized ones are Ubiquitin and SUMO-1 to -3. They are covalently and reversibly conjugated via isopeptide bond to the ϵ -side chain of lysines from thousands of proteins to regulate their function and fate. They are covalently and reversibly conjugated to lateral chains of lysines from thousands of proteins, of which they regulate the function and fate. Conjugation involves dedicated enzymatic cascades comprising an E1 UbL-activating enzyme (2 for Ubiquitin, 1 for SUMO), E2 UbL-conjugating enzymes (46 for Ubiquitin and 1 for SUMO) and several E3 factors (~700 for Ubiquitin and ~15 for SUMO). UbLs are involved in most cellular functions and are dysregulated in various pathologies, including cancer. In particular, the inventors have recently shown that the SUMO pathway plays an important role in AML response to standard chemotherapies (Bossis et al. 2014) and in the resistance of non-Acute Promyelocytic Leukemia AMLs to differentiation therapies by retinoic acid (Baik et al. 2018). Moreover, various E3 Ubiquitin ligases have been linked to AML chemoresistance. This is, for example, the case of IAPs (Inhibitors of Apoptosis) whose (i) overexpression is associated with poor prognosis and (ii) inhibition by "smacs mimetics" is currently being tested in clinical trials in various cancers,

including AMLs. Enzymes involved in UbL conjugation appearing frequently associated with AML resistance to treatments, the inventors have asked here whether assaying the deregulations of their activity might define a new class of easily implementable biomarkers of AML response to chemotherapies.

[0108] To this aim, the inventors first developed cellular models of resistance to Ara-C or DNR using two reference chemosensitive AML cell lines, HL-60 and U937. Generated Ara-C- (Ara-C-R) and DNR-resistant (DNR-R) sublines showed significantly higher IC₅₀ (**Figure 1**). To identify UbL substrate biomarkers of AML chemoresistance, the inventors then resorted to the Protoarrays from Life Technologies, which display more than 9000 recombinant human proteins spotted in duplicate on a nitrocellulose-coated slide. Such arrays have already been used successfully to identify substrates of certain E3 Ubiquitin ligases using either total cell extracts or recombinant enzymes. Identification of Ub- and SUMO-1 substrates and quantification of their modification were achieved by array scanning after incubation with, first, antibodies directed to either SUMO-1 or the Flag-tag epitope present on exogenous Ubiquitin and, then, fluorescent secondary antibodies (**Figure 2**). Three independent experiments were performed for all cell lines (24 arrays in total). Signals were corrected for background and normalized using the PAA package (Turewicz et al. 2016). Two different statistical analyses (Welch and Wilcoxon-Mann-Whitney) were then performed (**Figure 2**). This led to the identification of 988 ubiquitylated and 83 SUMOylated proteins (**Figure 3**).

[0109] The inventors then identified the proteins, which were differentially modified by the cell extracts from the resistant cell lines compared to the parental ones. A first analysis was performed by comparing the data for all Ara-C-R or DNR-R cells with those from parental HL60 and U937 cells. This led to the identification of 52 proteins differentially modified by Ubiquitin and 27 proteins differentially modified by SUMO-1 (**Figure 4**). The inventors then performed a second analysis in which the data for each cell line and each resistance (DNR and Ara-C) were considered separately. Although the sample size for each condition was lower, and thus the statistical significances lower than in the first analysis, the inventors identified 66 proteins for Ubiquitin and 15 for SUMO-1, which are differentially modified in at least one of the resistant cell lines compared to its parental counterpart (**Figure 5**). Altogether, the compilation of the inventors' data identified a modifomic signature of AML chemoresistance comprising 122 Ubiquitin/SUMO substrate proteins (**proteins of sequences SEQ ID NO: 1 to SEQ ID NO: 122**). An ontology analysis showed that these are principally involved in the Ubiquitin-proteasome pathway, stress responses, DNA damage repair and autophagy, which are all processes often dysregulated in chemoresistant cancer cells (**Figure 6**).

[0110] To further validate the signature, in particular on patient samples, the inventors

selected 23 of the 122 proteins that showed both a high level of modification and the most robust signal differences between sensitive and resistant cell lines (**proteins of sequences SEQ ID NO: 1 to SEQ ID NO: 23**). The inventors used them to generate a UbL score aiming at predicting patients' response to chemotherapy. Using this score, they could predict whether AML cell line were sensitive or resistant to DNR or Ara-C in 16 cases out of 18 tested (6 cell lines, 3 replicates) (**Figure 11**). To determine if it could also be used to predict AML patients' response to these drugs, they resorted to bone marrow aspirates from 4 patients, all of them being of the rather immature M1 subtype in the FAB classification. Two of these patients were sensitive to induction chemotherapy (anthracycline + cytarabine) and two were refractory. These samples were used to prepare extracts and to probe Protoarrays as described above for cell lines. Interestingly, the score could predict the response to chemotherapy of all of the 4 patients tested (**Figure 12**).

[0111] If Protoarrays are useful to identify modifomic signatures, they are not adapted for easy clinical diagnoses, as they require large numbers of cells, are expensive and difficult to standardize. Therefore, to prove on patient samples the prognosis value of the biomarkers the inventors identified, the inventors developed a miniaturized flow cytometry assay based on the use of Luminex XMap beads, which are color-coded magnetic beads. The inventors cloned 15 proteins of the signature (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19) in frame with GST, produced them in bacteria and coupled them to differently colored XMap beads. The coupled beads were then multiplexed (up to 10 different beads per tube) and incubated with extracts from parental-, Ara-C-R- or DNR-R HL60 cells before flow cytometry analysis using anti- SUMO-1 or Flag tag to quantify modification by SUMO and Ubiquitin, respectively (**Figure 7**). If ubiquitylation was detected on all of the proteins tested, this was unfortunately not the case for SUMOylation. A most likely explanation is that Ubiquitin can make long polyUbiquitin chains, facilitating its detection on protein substrates, whereas SUMO cannot.

[0112] Of the 10 proteins tested, four proteins (UBADC1, STAM, SQSTM1, and HIP2) showed significant differences in their ubiquitylation levels between parental, Ara-C- and/or DNR-resistant U937 (**Figure 13 and Figure 14**). Six out of the 15 tested proteins showed significant differences in ubiquitylation levels between parental and DNR-resistant HL-60 cells (**Figures 8 and 9**). The fact that not all proteins identified on the Protoarrays were validated in the XMap bead-based assay might be due to differences in their conformation and/or differences in pre-existing post-translational modifications, as they were produced in insect cells in the former case and bacteria in

the latter one.

- [0113] Finally, the inventors tested 13 of the UbL signature proteins with cell extracts from bone marrow aspirates of patients who responded, or not, to induction chemotherapy. Four of these proteins were found to be significantly ubiquitylated using patient cell extracts. Interestingly, these proteins showed a high level of ubiquitylation in 3/5 patients who did not respond to the induction chemotherapy but only in 2/12 patients who responded (**Figure 10** and **Table 2**). This means that the ubiquitylation of these 4 proteins, and probably others from the signature, might serve as therapeutic response biomarkers in AML.
- [0114] To further validate the use of the bead-based assay, the inventors used cell extracts prepared from bone marrow aspirates from 37 patients who responded, or not, to induction chemotherapy. They found that cell extracts obtained from patients who did not respond to induction chemotherapy resulted in higher levels of in vitro ubiquitylation of STAM, UBADC1, or SQSTM1 compared with those from patients who responded (**Figure 15**). 6 of 10 refractory patients showed high ubiquitylation levels for at least one of the biomarkers. In most cases, not all the biomarkers had high ubiquitylation levels, suggesting that the combination of biomarkers has higher predictive value than each biomarker alone. Altogether, this suggested that the ubiquitylation signature proteins, comprising among them STAM, UBADC1, and SQSTM1, might serve as a therapeutic response biomarker in AML.
- [0115] In conclusion, the inventors' work identifies a new class of modifomic biomarkers that might be used at diagnosis, in combination with other biomarkers and clinical data, to help clinicians predict AML patient response to anthracyclines and cytarabine. Importantly, the assay the inventors have miniaturized was shown adapted to patient sample analysis, paving the way to novel clinical developments.

Material and methods

Cell culture

- [0116] HL-60 and U937 cells (DSMZ, Germany) were cultured at 37 °C in RPMI medium supplemented with 10 % fetal bovine serum (FBS) and streptomycin/penicillin in the presence of 5 % CO₂. Both cell lines were authenticated by the ATCC using Short-Tandem-Repeat analysis. All cells were regularly tested negative for mycoplasma. After thawing, cells were passaged at a density of 3.10⁵/ml every 2-3 days for no more than 10 passages. U937 and HL-60 cells resistant to Ara-C (cytarabine) and DNR were generated by culture in the presence of increasing drug concentrations (up to 0.1 µM for Ara-C and 0.03 µM for DNR) for 2-3 months.

Patient samples

- [0117] Patient bone marrow aspirates were collected after obtaining written informed

consents from patients under the frame of the declaration of Helsinki and after approval by the institutional “Sud Méditerranée 1” Ethical Committee (ref 2013-A00260-45; HemoDiag collection). Fresh leukocytes were purified by density-based centrifugation using Histopaque 1077 (Sigma-Aldrich) and used immediately for extract preparation. Detailed characteristics and treatments of the patients involved in this study are provided in **Table 2**.

IC50 measurement

- [0118] Cells were seeded at a concentration of $2 \cdot 10^5$ /mL in RPMI medium complemented with 0.1, 1, 10, 50, 100 or 250 μ M of Ara-C (Sigma-Aldrich) or 0.01, 0.05, 0.1, 0.5, 1 or 10 μ M of daunorubicin (Sigma-Aldrich). Viability was measured 24 hrs later using the MTS assay from Promega following the manufacturer's protocol. IC₅₀ were calculated using the GraphPad PRISM software.

Cellular extracts

- [0119] Cells grown at a $5\text{--}8 \cdot 10^5$ /mL density were spun down (300 g) at 4 °C for 5 min and washed once with PBS. After pellet resuspension in 1 mL of PBS, they were centrifuged again (16,000 g) at 4 °C for 5 min. Pellets were resuspended and incubated at 4 °C for 30 min in a hypotonic buffer (20 mM HEPES pH 7.5, 1.5 mM MgCl₂, 5 mM KCl, 1 mM DTT and 1 mg/L of aprotinin, leupeptin and pepstatin) in a volume of either (i) 100 μ L per $50 \cdot 10^6$ cells to generate concentrated extracts used for ProtoArray probing or (ii) 25 μ L per $2 \cdot 10^6$ cells in the case of extracts used in XMap bead-based flow cytometry assays. Cell lysis was achieved through 4 freezing/thawing cycles using liquid nitrogen and DNA was sheared owing to 10 passages through a 20-1/2G needle. Extracts were finally centrifuged twice (16000 g) at 4°C for 20 min and supernatants were aliquoted, flash-frozen and kept at -80 °C until use.

Protein arrays

- [0120] Human protein arrays (ProtoArrays from Life Technologies) were equilibrated at 4 °C for 15 min and, then, saturated at 4 °C for 1 hr with the Protoarray-Blocking Buffer (Life Technologies) containing both the Synthetic Block from Life Technologies and 1 mM DTT. To probe ProtoArrays for protein modification by UbLs, cell extracts (concentrated) were first supplemented with 5 μ M Ubiquitin-Vinyl sulfone, 2.5 μ M SUMO1-Vinyl sulfone, 2.5 μ M de SUMO2-Vinyl sulfone and 2.5 μ M NEDD8-Vinyl sulfone (Boston Biochem) to inhibit UbL-deconjugating enzymes. Control extracts were also incubated with 50 mM N-Ethyl Maleimide (NEM; Sigma-Aldrich) to inhibit any UbL conjugation. The extracts were then supplemented with home-made recombinant SUMO-1 and SUMO-2 (15 μ M, produced as previously described), 30 μ M Flag-ubiquitin (Boston Biochem), 15 μ M NEDD8 (Boston Biochem), 0.1% Tween-20, and 1 mM ATP. Immediately after, they were laid on ProtoArray slides, which were

covered with a coverslip and incubated at 30 °C for 1 hr in a humidified atmosphere. Arrays were washed 3 times for 5 min with a washing buffer (PBS pH 7.4, 0.1 % Tween 20, 1X Synthetic Block) supplemented with 0.5 % SDS and, then, twice for 5 min with only the washing buffer. Next, they were incubated under agitation (50 rpm) at 4°C for 1 hr with an anti-Flag- (M2 antiserum from SIGMA) and an anti-SUMO-1 (21C7 from the Developmental Studies Hybridoma Bank) antibody at a 1 µg/mL concentration in the washing buffer. Then, they were washed 5 times for 5 min in the washing buffer and incubated at 4 °C for 90 minutes with Alexa Fluor 647-labelled anti-mouse- and Alexa Fluor 546-labelled anti-rabbit (Thermo Fisher) antibodies at a 0.5 µg/mL concentration. Arrays were washed 5 times for 5 min with the washing buffer, once with H₂O and, finally, dried by centrifugation before fluorescence scanning using the Innoscan 710 device from Innopsys. The arrays were then stripped with 200 mM Glycine, 0.5% SDS, pH 2.2 for 20 min and probed again with anti-SUMO-2 (8A2 from the Developmental Studies Hybridoma Bank) and anti-Nedd8 (Y297, Abcam) antibodies. However, some signals were remaining from the first hybridization. To avoid false positive, the inventors thus decided to consider only the signals obtained for SUMO-1 and Ubiquitin.

Analysis of Protoarray data

[0121] Measured fluorescence intensities were associated to the corresponding protein ID according to their coordinates on the arrays using the Mapix software (Innopsys). Intensities were processed using the PAA R package. In brief, (i) duplicated protein spot intensities were averaged using the Load GPR function and (ii) the background was corrected using the Background Correct function. Fluorescence intensities within the same experiment were normalized by quantiles using the Normalize Array function. Finally, intensities were normalized between the different experiments using the Batch Adjust function.

Data filtering

[0122] To identify the proteins modified by Ubiquitin or SUMO-1 on the ProtoArrays, the inventors first had to filter proteins displaying signal significantly higher in UbL conjugation-permissive- than those in UbL conjugation non-permissive conjugation conditions (i.e. control conditions using NEM-treated extracts). With regard to the statistical test adapted to the exploitation of the inventors' results, the classical Student t-test was not recommended, as variances could be very different between UbL conjugation permissive and non-permissive conditions. The inventors therefore selected proteins with significant p-values (lower than 0.05) in both the parametrical Welch- and the non-parametrical Wilcoxon-Mann-Whitney (WMW) test. Then, proteins having an averaged normalized fluorescence intensity value less than 800

were filtered out in order to obtain a list of robustly modified proteins.

[0123] **Identification of proteins differentially UbL-modified between chemosensitive and chemoresistant cells**

[0124] As the fluorescence intensity signals obtained cannot be considered as precisely quantitative and to work on more robust values, the inventors decided to work on ratios between the parental and drug-resistant cell samples for each protein. The inventors thus compared, for each of the modified proteins (NEM-filtering), the ratios between sensitive and resistant cells using both a Wilcoxon signed-rank test and a one sample *t*-test. This analysis was first performed on all arrays (both cell lines and drugs combined). In this case, the sample size being large, the inventors considered as differentially modified all proteins showing $p\text{-values} < 0.05$ in both tests. For the analysis on the individual cell lines (U937 and HL-60) and drugs (Ara-C and DNR), the sample size being smaller, the inventors considered as differentially modified the proteins showing $p\text{-values} < 0.05$ in the one sample *t*-test.

[0125] **Generation of a predictive score using a Genetic Algorithm and Linear Discriminant Analysis**

[0126] The R package GA, providing a genetic algorithm (GA), was used. The aim was to determine the right number of variables in order to create a parsimonious predictive model. GAs are mathematical models inspired by Charles Darwin's model of natural selection. The natural selection preserves only the fittest individuals over the different generations. An evolutionary algorithm improves the selection over time and allows the best solution to emerge from the best of prior solutions. The selected features were then tested with linear discriminant analysis (LDA) using the R package MASS. This mathematical method uses a linear combination of all variables to assign observations to target classes. To this aim, it creates a decision rule based on the n available variables ($\text{score} = \beta_1 + \beta_2 + \dots + \beta_n$) by maximizing the between-class variability and minimizing the within-class one. A cross-validation step that separates the observations in two groups (training dataset on which the model is established and test dataset on which the model is validated) was performed to get more robust results.

Production of recombinant proteins

[0127] cDNA encoding for the proteins of interest were recovered from the Ultimate ORF library (ThermoFisher) and cloned in the bacterial expression vector pGGWA vector using the Gateway technology according to manufacturer's protocol (Life Technologies). Constructs were then transformed in BL21 (DE3) *E. coli* strain. Protein production was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 6 hrs in exponentially growing bacteria at 25°C.

[0128] Bacterial pellets were resuspended in 50 mM Tris-HCl pH 8.6 containing 500 mM NaCl and 50 mM MgSO₄, and flash-frozen in liquid N₂. After thawing, bacterial sus-

pensions were supplemented with 1 mg/mL lysozyme (Sigma-Aldrich), 8 mM β -mercaptoethanol, 1 mg/L aprotinin, leupeptin and pepstatin and incubated at 4°C for 1 hr. Bacterial debris were spun down (100 000 g for 1 hr). The extract was then bound to Glutathion agarose beads (Generon) equilibrated in Tris 50 mM pH 8.6, NaCl 500 mM, MgSO₄ 50 mM, 8 mM β -mercaptoethanol, 1 mg/L aprotinin, leupeptin, pepstatin. The column was then extensively washed with Tris 50 mM pH 8.6, NaCl 150 mM, MgSO₄ 50 mM, 8 mM β -mercaptoethanol, 1 mg/L aprotinin, leupeptin, pepstatin and eluted by addition of 20 mM reduced glutathione (Sigma-Aldrich).

Protein coupling to XMap

- [0129] 2.10⁵ magnetic XMap beads (low concentration) from Luminex were transferred to a low binding microtube (Eppendorf) and washed using 500 mM NaCl. They were then resuspended in 50 μ L of 50 mM MES pH 6.1 and incubated in the presence of 5 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) and 5 mg/mL Sulfo-NHS (Pierce) at room temperature for 20 min. Beads were then washed in PBS containing 500 mM NaCl and incubated with 7 μ g of recombinant protein to be coupled in 100 μ L PBS at room temperature for 2 hrs. They were then washed twice with PBS containing 0.1 % BSA, 0.02 % Tween 20, 0.05 % sodium azide and 500 mM NaCl and stored at 4 °C in PBS containing 0.1 % BSA, 0.02 % Tween-20, 0.05 % sodium azide.

UbL conjugation to proteins coupled to XMap beads

- [0130] SUMO-1-, SUMO-2- and Ubiquitin vinyl sulfones (0.5 μ M each) were added to diluted cellular extracts (10 μ L), which were incubated at 4 °C for 15 min. Control extracts were also incubated with 50 mM NEM. The inventors then added to the extract 10³ protein-coupled XMap beads contained in 10 μ L of a reaction buffer containing 20 mM HEPES pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)₂, 0.05 % Tween-20, 0.5 mM EGTA, 0.2 mg/mL ovalbumine, 1 mM DTT, 1 mg/L aprotinin, leupeptin and pepstatin, 1 mM ATP, 30 μ M Flag-ubiquitin, 15 μ M SUMO-1 and 15 μ M SUMO-2. Reaction were performed at 30 °C for 45 min. Beads were washed twice for 5 min with PBS containing 0.05 % Tween-20 and 0.5 % SDS and 3 times for 5 min with PBS containing 0.05 % Tween-20. They were then incubated with 1 μ g/mL of anti-SUMO-1 (21C7) and anti-Flag antibodies for 1 hr under agitation at room temperature. After washing in PBS containing 0.05% Tween-20 for 5 min, they were incubated for 30 min at room temperature with anti-mouse Alexa Fluor 488- and anti-rabbit Alexa Fluor 405 antibodies in 100 μ L of PBS containing 0.05 % Tween-20. Beads were again washed for 5 min with PBS containing 0.05 % Tween-20. They were then resuspended in 200 μ L PBS and flow-cytometry-analysed using the LSR Fortessa device from BD Biosciences. Results were analysed using the FlowJow software.

[0131] **Table 2** : Age* = Age at diagnosis; Blasts* = Blasts at diagnosis in bone marrow;
 [0132] Blasts** = Blasts 30 days after diagnosis in bone marrow

[0133]

ID	Sex	Age *	FAB	Blasts*	Treatment	Blasts**	Karyotype	Molecular biology
15085	F	41	AML 1	78%	Daunorubicin 3 Cytarabine 7	5%	46,XX,t(11;19)(q23;p13.1)	MLL rearrangement
15088	F	46	AML 1	72%	Daunorubicin 3 Cytarabine 7	3%	46, XX, t(12;20) (q24;q13) (constitutional translocation)	CEBPA double mutation
15096	M	25	AML 5	88%	Idarubicin 5 Cytarabine 7	3%	46, XY, t(11;22) (q23;q11.2)	MLL rearrangement
16122	M	74	AML 2/4	45%	Idarubicin 5 Cytarabine 7 Lomustine	1%	46,XY	
16137	M	63	AML 2	62%	Idarubicin 5 Cytarabine 7 Lomustine	2%	46, XY	NPM1 A+
16152	F	54	AML 4	48%	Idarubicin 5 Cytarabine 7 Lomustine	2%	46, XX	Mutation NPM1-A
16174	M	68	AML 2	24%	Idarubicin 5 Cytarabine 7 Lomustine	5%	45, XY,-7	
16185	F	69	AML 1	68%	Idarubicin 5 Cytarabine 7 Lomustine	1%	Complex karyotype, hypodiploid with 39 chromosomes, monosomy of chromosome 7 and deletion 5q	
17235	F	58	AML 2	34%	Idarubicin 5 Cytarabine 7	1%	47, XX,+mar (constitutional)	Duplication FLT3-ITD Mutation NPM1-A

[0134]

ID	Sex	Age *	FAB	Blasts*	Treatment	Blasts**	Karyotype	Molecular biology
18294	F	62	AML 4 Eo	55%	Idarubicin 5 Cytarabine 7	2%	47,XX,+8,inv(16)(p13q22)	CBFB-MYH11
18309	F	39	AML 2	60%	Idarubicin 5 Cytarabine 7	1%	46,XX	CEBPA double mutation
18299	M	61	AML 2	38%	Idarubicin 5 Cytarabine 7 Lomustine	1%	45,XY,-7	
15098	M	37	AML 0	94%	Daunorubicin 3 Cytarabine 7	95%	46,XY, t(6,14)(q21;q32), +4	
15082	F	72	AML 4	56%	Idarubicin 5 Cytarabine 7 Lomustine	24%	49, XX, +8, +21, +22(10)	
17217	F	42	AML 2	50%	Idarubicin 5 Cytarabine 7	52%	Complex karyotype, monosomal hypodiploid with 44 chromosomes, rearrangement of chromosome 3, and monosomy of chromosome 7 and 17. Rearrangement of EVI1	
18298	F	68	Biphenotypic AL (AML + ALL B)	86%	Idarubicin 5 Cytarabine 7 Lomustine	70%	45, XX, -7, inv(11;3) (q21;q21q26)	
17202	F	67	AML 0/1	27%	Idarubicin 5 Cytarabine 7 Lomustine	25%	43 XX,-5,-7,- 12,add(12)(p13),add(16)(q24),- 17,+mar(8)	

Claims

[Claim 1]

A method for determining *in vitro* drug-resistance of cells of a leukemic sample obtained from a patient afflicted by a leukemia, said method comprising:

- a. a step of evaluating, from cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins;

wherein said at least 4 proteins correspond to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins correspond to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,

- a. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said at the least 4 proteins of cells in said leukemic sample corresponding to a reference level, said reference level being the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins of leukemic cells that are sensitive to said drug in order to obtain a ratio of the level in the leukemic sample compared to the reference level of a sample of sensitive cells for each of said at least 4 proteins

said reference level corresponding to the mean of the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins of several, possibly independent, leukemic cells that are sensitive to said drug; and

- a. a step of determining that
 - if the ratio for at least one of said 4 proteins is higher than 1.25 or lower than 0.8, then leukemic sample will be resistant to said drug, and

- if the ratio for each of said at least 4 proteins is comprised from 0.8 to 1.25, then leukemic sample will be sensitive to said drug.

- [Claim 2] The method according to claim 1, wherein
- the protein consisting essentially or consisting of the sequence as set forth in SEQ ID NO: 1 is modified by Sumoylation,
 - the protein consisting essentially or consisting of the sequence as set forth in SEQ ID NO: 2 is modified by Ubiquitination, and
 - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 3 and SEQ ID NO: 4 are modified by Ubiquitination and Sumoylation.
- [Claim 3] The method according to claim 1 or 2, wherein if the modification of the protein as set forth in SEQ ID NO: 2 or 3 or 4 is an ubiquitination, and if the ratio is higher than 1.25, then leukemic sample will be resistant to said drug.
- [Claim 4] The method according to claim 1 or 2, wherein if the modification of the protein as set forth in SEQ ID NO: 1 or 3 or 4 is a sumoylation, and if the ratio is higher than 1.25, then leukemic sample will be resistant to said drug.
- [Claim 5] The method according to anyone of claims 1 to 4, comprising a step a) of evaluating, in said leukemic sample, the level of modification by ubiquitination and/or sumoylation of each protein of said group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins.
- [Claim 6] The method according to anyone of claims 1 to 5, wherein
- the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 1, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 20 are modified by Sumoylation,
 - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 2, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 17 to 19, SEQ ID NO: 21 and SEQ ID NO: 23 are modified by Ubiquitination, and
 - the proteins consisting essentially or consisting of the respective sequences as set forth in SEQ ID NO: 3 and 4, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 16 and SEQ ID NO: 22 are modified by Ubiquitination and Sumoylation.

- [Claim 7] The method according to anyone of claims 1 to 6, comprising a step a) of evaluating, in said leukemic sample, the level of modification by ubiquitination and/or sumoylation of each protein of said set of 122 proteins.
- [Claim 8] The method according to anyone of claims 1 to 7, wherein said drug is chosen from anthracyclin and/or cytarabine.
- [Claim 9] The method according to anyone of claims 1 to 8, wherein said leukemia is an acute myeloid leukemia.
- [Claim 10] A method for the in vitro prognosis of a leukemia in a sample of a patient comprising the following steps:
- a. a step of evaluating, from cells of said leukemic sample, the level of modification by ubiquitination or sumoylation of each protein of at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins;

wherein said at least 4 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,

- a. a step of comparing the level of modification by ubiquitination and/or sumoylation of each said at the least 4 proteins in cells of said leukemic sample to the level of modification by ubiquitination and/or sumoylation of each at the least 4 proteins, in leukemic samples that are sensitive to said drugs, in order to obtain a ratio of the level in the leukemic sample compared to a panel of drug-sensitive-leukemic samples for each of at least 4 proteins; and
 - b. a step of determining that if the ratio of each of said at least 4 proteins is comprised from 0.8 to 1.25, then the patient could be treated with anthracyclin and/or cytarabine.
- [Claim 11] The method according to claim 10, wherein in step c) if the ratio is lower than 0.8 or higher to 1.25 for at least one of said at least four

proteins, then the patient should be treated with FLT3, IDH1/2, Blc2 proteins inhibitors or enrolled in a clinical trial for new molecules.

[Claim 12]

A kit comprising:

- at the least 4 proteins chosen among a group of 23 proteins, said group of 23 proteins belonging to a set of 122 proteins, wherein said at least 4 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 4, wherein said 23 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 23, and wherein said group of 122 proteins corresponds to the proteins consisting essentially or consisting of the sequences SEQ ID NO: 1 to 122,
- at least one antibody directed against Ubiquitin and at least one antibody directed against SUMO, and
- means for detecting the ubiquitination and/or sumoylation of said at least 4 proteins.

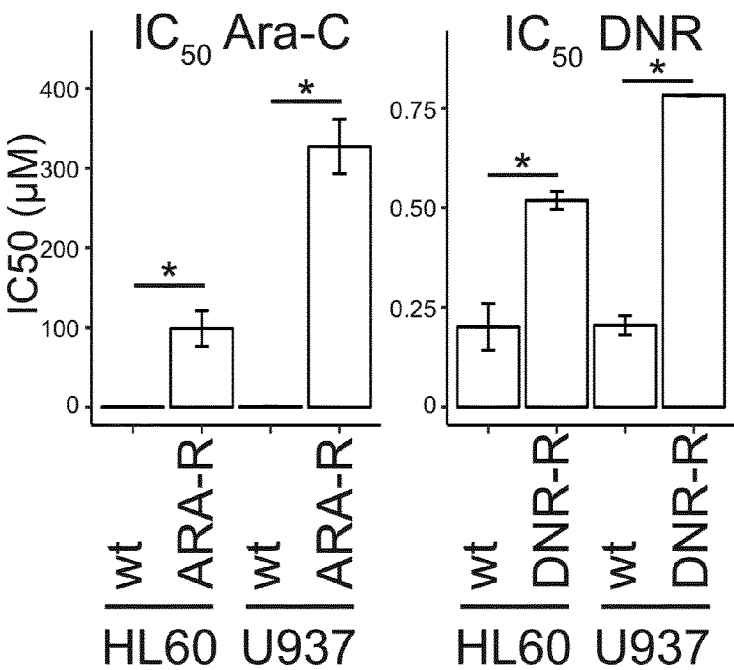
[Claim 13]

The kit according to claim 12, further comprising leukemic cells, or extracts of leukemic cells that are sensitive to a determined drug.

[Claim 14]

The kit according to claim 14, wherein said determined drug is anthracyclin and/or cytarabine.

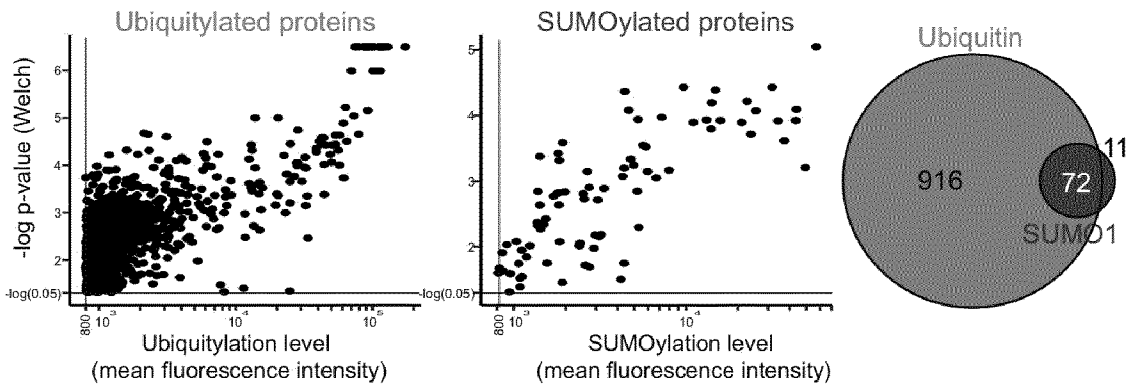
[Fig. 1]



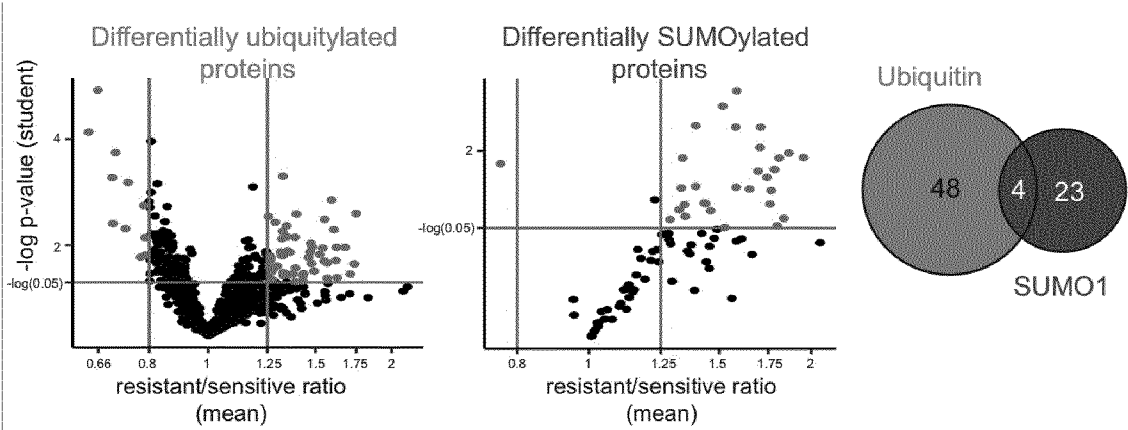
[Fig. 2]

extract	+	reaction	revelation	analysis
HL60 HL60-ARA-R HL60-DNR-R	UbL-VS UbL	Protoarray	1 st antibodies (SUMO-1 and Flag)	normalization (PAA)
U937 U937-ARA-R U937-DNR-R	(NEM for control slide)		2 nd antibodies (mouse-647 rabbit-546) scan	arrays comparison signature

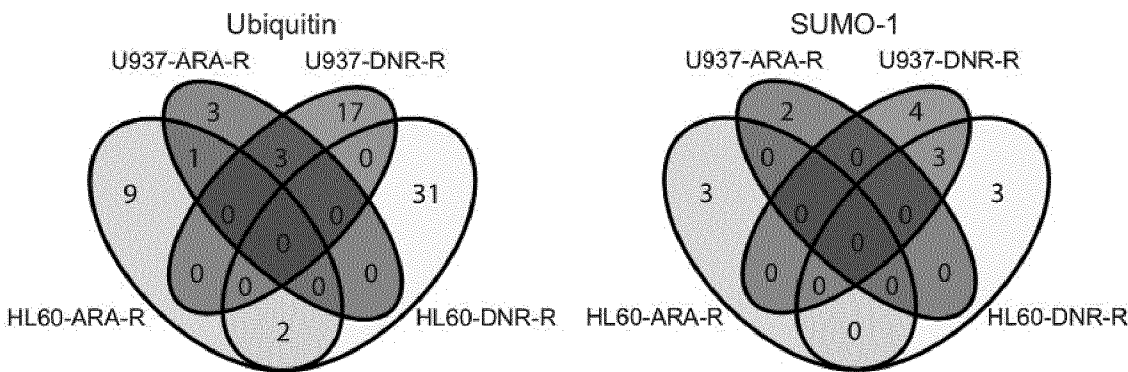
[Fig. 3]



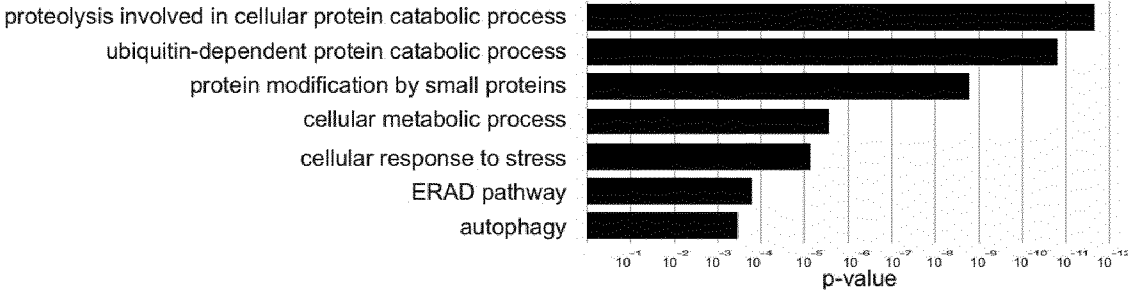
[Fig. 4]



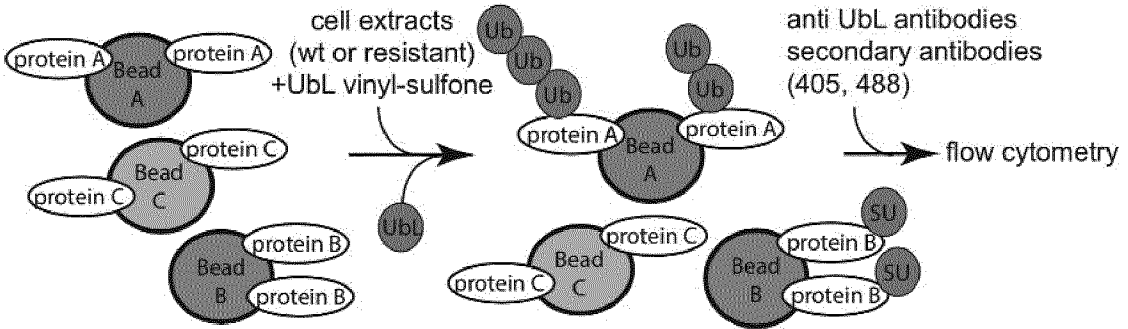
[Fig. 5]



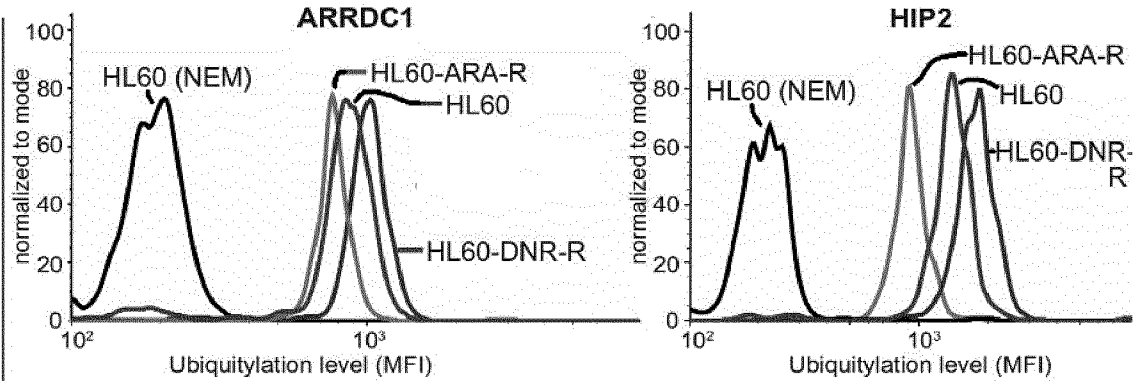
[Fig. 6]



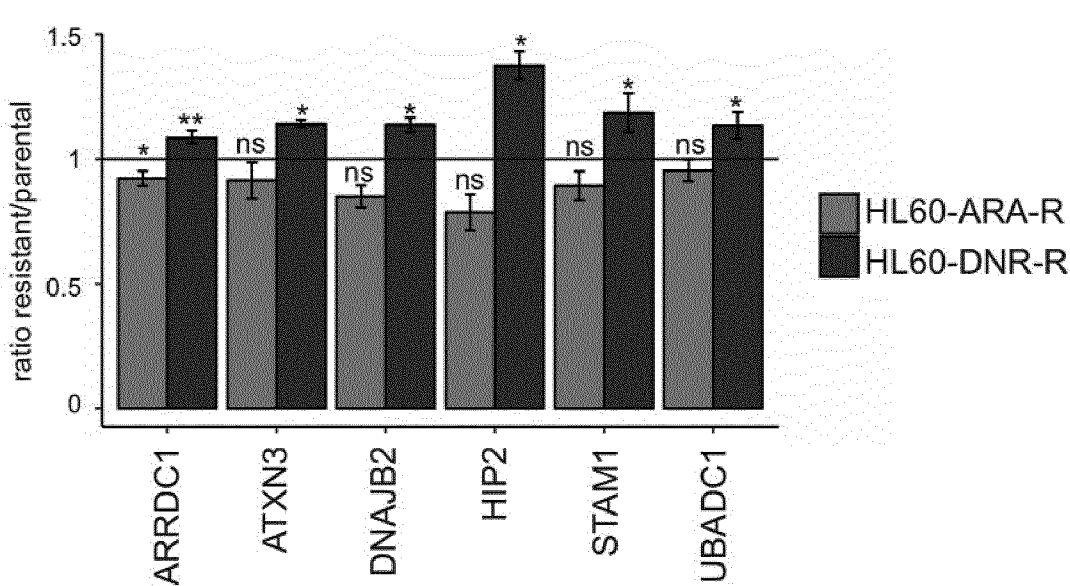
[Fig. 7]



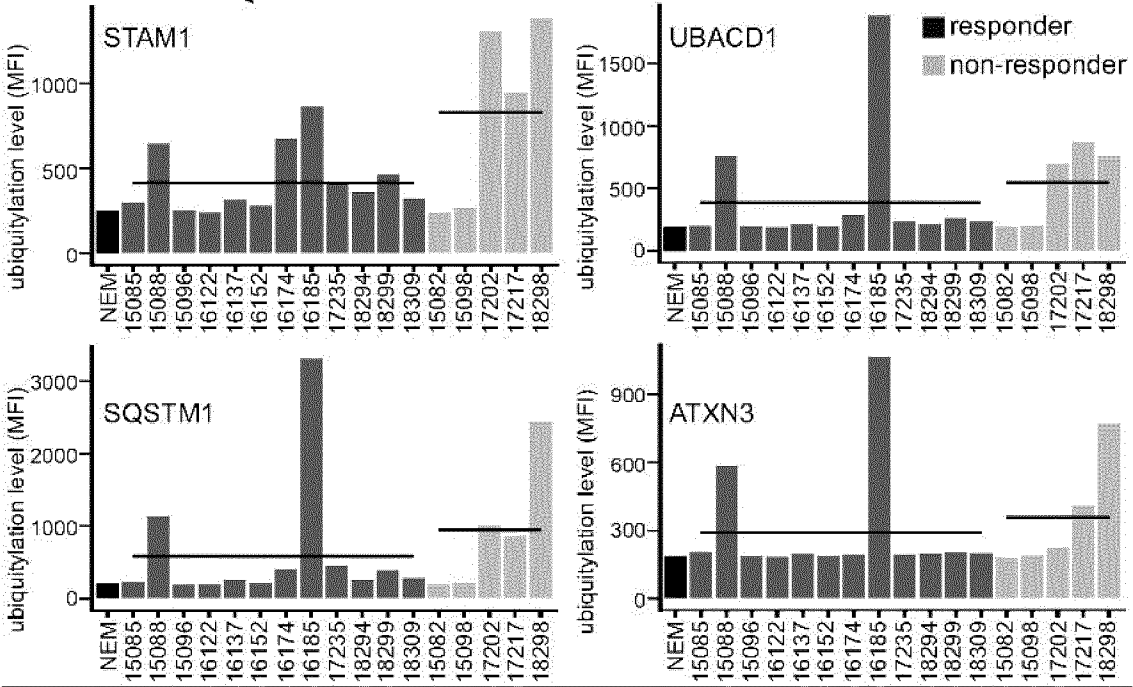
[Fig. 8]



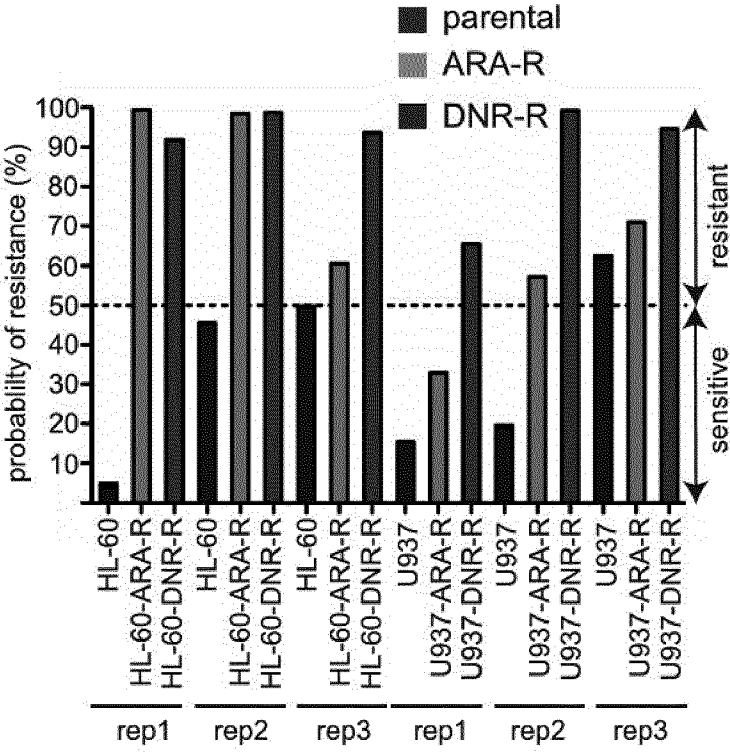
[Fig. 9]



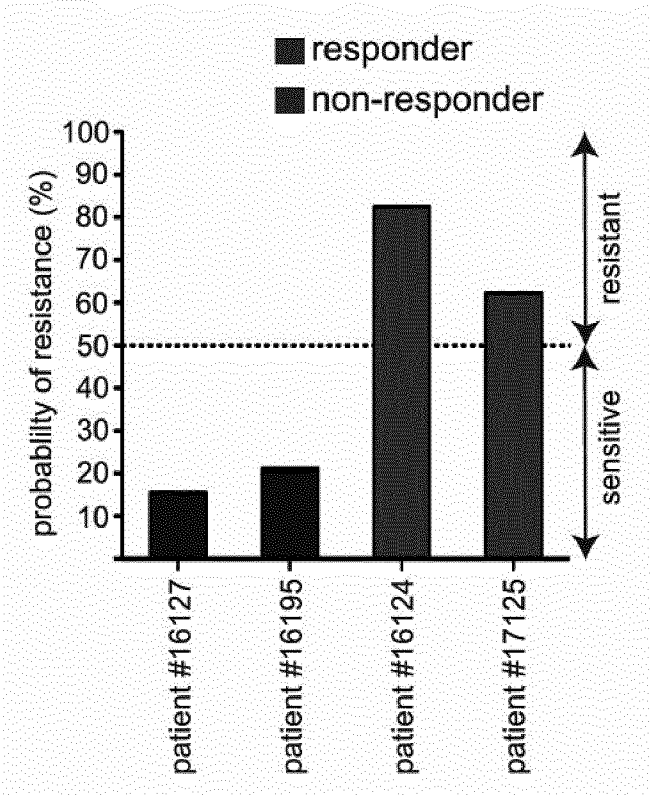
[Fig. 10]



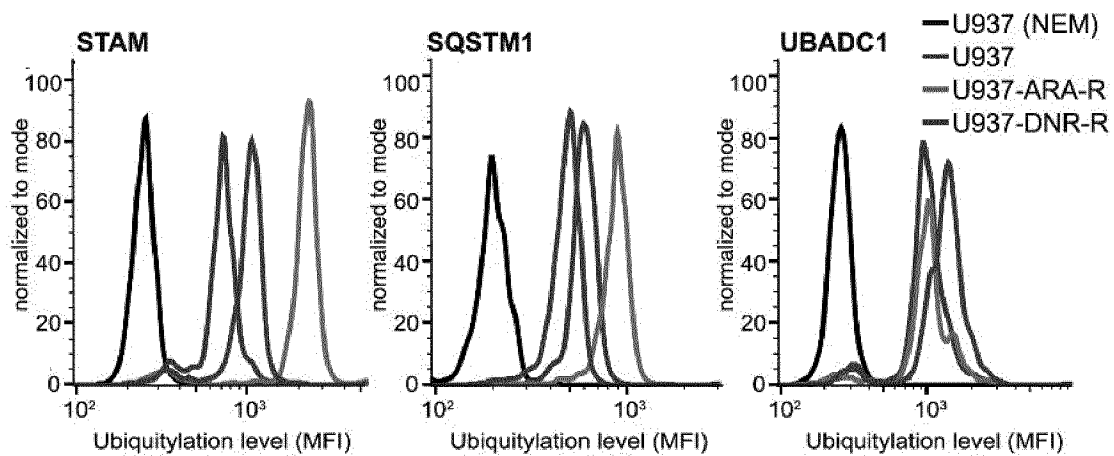
[Fig. 11]



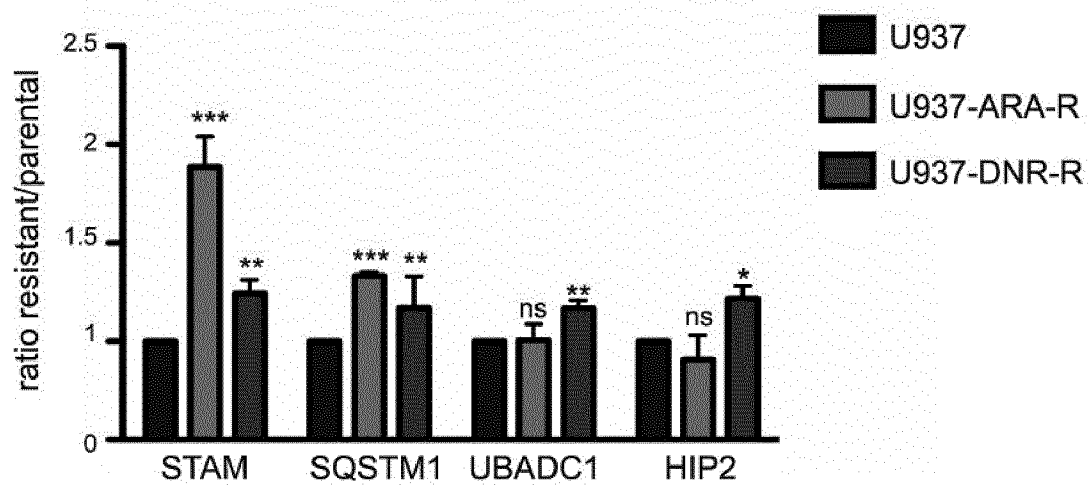
[Fig. 12]



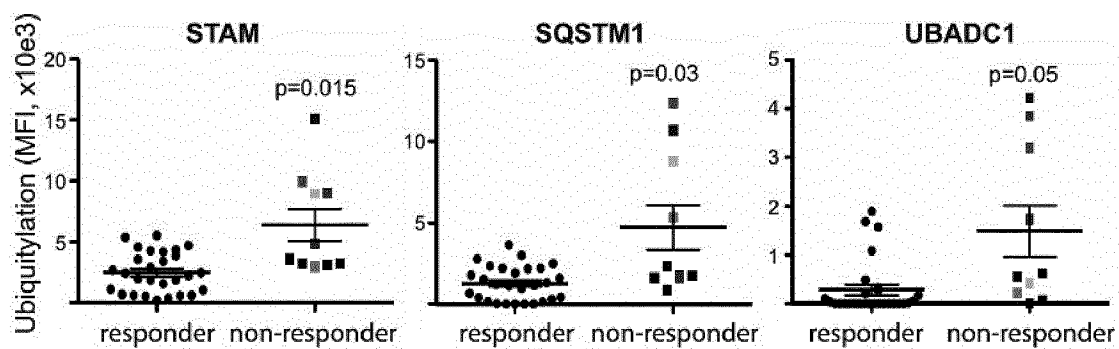
[Fig. 13]



[Fig. 14]



[Fig. 15]



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/064909

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/34 G01N33/574
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 G01N

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DIJIONG WU ET AL: "Autophagy and Ubiquitin-Mediated Proteolytic Degradation of PML/Rar[alpha] Fusion Protein in Matrine-Induced Differentiation Sensitivity Recovery of ATRA-Resistant APL (NB4-LR1) Cells: in Vitro and in Vivo Studies", CELLULAR PHYSIOLOGY AND BIOCHEMISTRY., vol. 48, no. 6, 1 January 2018 (2018-01-01), pages 2286-2301, XP055647006, CH ISSN: 1015-8987, DOI: 10.1159/000492646 the whole document abstract page 2287 - page 2299; figures 1-6 ----- -/-	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

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

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Date of the actual completion of the international search

9 June 2020

Date of mailing of the international search report

17/06/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Authorized officer

Boiangiu, Clara

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/064909

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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